IMMUNITY IN KWASHIORKOR

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Thesis Submitted for the

Degree of Doctor of Medicine

University of Cape Town March 1980

ABSTRACT

The adverse effects of malnutrition on the child are diverse and complex. This thesis describes an examination of the effects of severe malnutrition on immune defence mechanisms and in particular on cellular immune function *in vitro*. The contents and conclusions of the chapters may be summarised as follows:-

A review of the literature makes it clear that malnutrition predisposes the child to an increased frequency of infections. When children with severe protein energy malnutrition contract infections, the pattern of disease which follows is both more severe and is subject to the development of serious complications. The overall effect is that the combination of malnutrition and infection leads to an increased morbidity and mortality. There is considerable evidence to indicate that protein energy malnutrition exerts a deleterious effect on cell-mediated immune responses. Lymphoid organs are atrophied, *in vivo* delayed hypersensitivity responses are blunted and *in vitro* functional responses of mitogen-stimulated lymphocytes are deficient. In many respects the clinical and experimental findings in these children are analogous to the findings in children suffering from primary cellular immune deficiency disorders.

Although the major demonstrable effects of malnutrition on the immune response have been ascribed to depression of T lymphocyte function, there is also evidence that other aspects of immunity are affected. Abnormalities of humoral immunity, complement function and phagocytic cell activity have all been described. There is thus compelling reason to believe that the immune status of the malnourished child is defective and that this contributes to increased morbidity and mortality rates.

Although the lymphocyte transformation test has been widely used and reported as a valid test of cellular immune function because of its functional nature, it is subject to wide variations. Factors affecting this test have been examined in detail and as a result it has been possible to fully standardise and control aspects of this assay. In certain areas this has not been completely achieved, but variation has been reduced by accurate counting of lymphocytes; by standardising control serum and control lymphocytes as far as possible; and by attention to the procedures for the separation and isolation of lymphocytes from peripheral blood.

Thirty six children with kwashiorkor or marasmic kwashiorkor and 10 normal children were investigated. Clinical, radiological or bacteriological evidence of infection was present in 33 of the 36 children with kwashiorkor. These children had elevated levels of IgG, IgM, IgA and IgE. During treatment a further elevation of IgG, IgM and IgA levels was apparent. However, IgM and IgA levels declined in the latter part of the treatment period but were still elevated after 6 weeks when compared with values obtained in control children. Blood group isohaemagglutinin levels were normal in the kwashiorkor children. Antibody production following immunisation was not examined in this study but the further rise in immunoglobulin levels on refeeding suggests that there is an increased capacity for antibody production following treatment.

Low serum C3 levels were present in these children with kwashiorkor. During nutritional recovery serum C3 levels rose to supranormal values and then fell back into the normal range by 6 weeks. These findings confirm the observation that the complement system is depressed in severe undernutritional states but are unable to answer the question of whether this is due to increased consumption or decreased synthesis

of complement.

The normal results obtained with the nitro-blue tetrazolium test in these children indicate that some aspects of polymorphonuclear phagocytic cell function are intact. Reference to the literature, however, shows that a more detailed investigation of this aspect of immunity is able to demonstrate a number of defects.

This thesis confirms and extends the observations of others that children with kwashiorkor have grossly abnormal cellular immune function. Depressed lymphocyte counts, poor delayed hypersensitivity skin responses to dinitrochlorobenzene, PPD and Candida, and reduced peripheral blood T cell numbers were observed in these patients.

This study shows that lymphocyte responses to phytohaemagglutinin (PHA) were consistently diminished in cultures supplemented with kwashiorkor sera. This depressed response was seen in both autologous lymphocyte cultures and in cultures of normal reference lymphocytes. The serum of normal children supported the transformation of control cells and reference cells in a normal manner. This serum effect was not mitogen-specific inasmuch as it affected blastogenic responses to PHA, Con A and PWM and was even more striking in mixed lymphocyte cultures. With nutritional recovery the serum effect on PHA-stimulated and mixed lymphocyte cultures returned to normal. This return to normal values was delayed, however, when compared with serum albumin and globulin fractions which had attained normal values after two weeks of treatment.

On the basis of a statistical analysis of PHA-induced mitogenic responses in AB serum, intrinsic lymphocyte function in kwashiorkor did not appear to be abnormal. Examination of the responses in individual cases, however, showed that up to one third of the patients' lymphocytes showed abnormally low responses in AB serum. Therefore, although the

serum effect was consistently present in all cases, some kwashiorkor patients have an intrinsically defective lymphocyte responsiveness in normal serum which returned to normal following nutritional recovery.

The magnitude of the serum defect in lymphocyte transformation could not be clearly correlated with the degree of malnutrition, the presence or absence of infection or other laboratory manifestations of kwashiorkor.

Lectin dose-response experiments showed that the kwashiorkor serum effect did not act by inactivating or otherwise reducing the effective concentration of the mitogen. Kwashiorkor serum supplements gave inadequate blastogenic responses, not only as measured by $[^{14}C]$ -thymidine incorporation into white cells but also as measured by nuclear sizing techniques and histological evaluation of autoradiographs.

Definitive mixing experiments showed that the defect in kwashiorkor serum was due to a deficiency of some factor(s) essential for optimal lymphocyte transformation and not due to the presence of an inhibitor. The results of viable cell counts, the reversibility of the kwashiorkor serum effect and the results of serum mixing experiments exclude the possibility that kwashiorkor serum was simply cytotoxic for lymphocytes. Serum switch experiments indicated that optimal transformation required the presence of normal serum throughout both the initial and late phases of the mitogenic response. Cultures pulsed with $[^{3}H]$ -uridine and $[^{3}H]$ -thymidine indicate that synthesis of both RNA and DNA was affected by the inadequacy of the kwashiorkor serum and it is therefore possible that kwashiorkor serum effects on DNA synthesis are secondary to its effect on prerequisite synthesis of RNA or other macromolecules.

The precise chemical nature of the compound(s) that is (are) lacking in kwashiorkor serum has not as yet been defined. This could

to a large extent be supplied by dialysis of deficient serum against AB serum and by the addition of an ultrafiltrate of AB serum, indicating that kwashiorkor serum was mainly deficient in some low molecular weight (< 500 daltons) component(s) required for optimal transformation. The addition of albumin did not improve the supportive quality of kwashiorkor serum.

Since the kwashiorkor serum deficiency was manifest in the presence of various tissue culture media, the low molecular weight component(s) that were lacking were not among those amino acids, vitamins, salts, energy substrates or other known growth factors normally included in the formulation of such media.

Skin fibroblasts, although initially retarded in the *in vitro* proliferation in the presence of kwashiorkor serum, were able to make good the deficiency by "conditioning" their media. Lymphocytes appear to lack this capacity.

Experiments conducted with the ultrafiltrate of normal serum have not been helpful in defining the biochemical characteristics of this deficient substance. The active property (ies) is (are) acid-labile and can be destroyed by heating to 500°C. Preliminary extraction procedures suggest that methanol extraction may be the most promising means of concentrating and identifying this low molecular weight substance.

Although serum factors that support lymphocyte transformation are complex and their positive and complete identification represents a formidable task, it is hoped that this final characterisation will be achieved. The availability of such compounds in a pure biologically active form would contribute much to the understanding and management of the immunodeficiency of kwashiorkor.

ACKNOWLEDGEMENTS

The work for this thesis was undertaken in the Department of Clinical Science and Immunology at the University of Cape Town's Medical School and was supervised by Professor Eugene Dowdle. His inspiration, intellect and enthusiasm have been a constant source of help and encouragement to me and I consider myself most fortunate in having had the opportunity to study and learn under his expert guidance. I am also indebted to many members of the Department of Clinical Science and Immunology for their practical guidance and their example of excellence, which is a characteristic of the scientific research undertaken by the Department.

The children described in this thesis were patients at the Red Cross War Memorial Children's Hospital. I am most grateful to Professor Malcolm Bowie for allowing me unlimited access to the facilities of the Metabolic Unit in this hospital and to the medical and nursing staff for their assistance in the clinical management of these patients.

I would like to thank the following people and organisations for generously providing me with the services, assistance and facilities required during this study:-

- Mrs Sue Gardner whose professional technical assistance was invaluable in the performance of the lymphocyte function assays.
- The children and their parents who willingly cooperated in the study.
- The staff and blood donors of the Western Province Blood Transfusion Service who ensured a regular supply of blood for the isolation of normal lymphocytes and normal sera.

- The Medical Officer of Health for Cape Town and the nursing staff of the Heideveld Child Welfare Clinic who assisted me in gaining access to normal children.

- Mr Mike Waring who designed and built the multiple automatic sample harvester.

- Dr Ann Orren and Mr Wolfgang Lerch who performed the IgE estimations.

- Miss Christine Fraserman who performed the serum protein electrophoresis measurements.

- The Pathology laboratories at the Red Cross Children's Hospital who undertook the routine haematological and bacteriological investigations.

- The South African Medical Research Council who provided generous financial support for this work.

- Mrs Annemarie Garschagen who painstakingly typed this thesis and provided expert advice on the final draft.

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INTRODUCTION

In many parts of the world, the two most important factors affecting the health of young children are malnutrition and infection. The exact prevalence of severe malnutrition is difficult to assess but experience suggests that it affects a significant fraction of the population. The available literature which covered 80 surveys in 39 countries has been reviewed and this shows that in children under the age of 5 years the point prevalence of severe malnutrition is between 0 and 7,6%, while for moderate malnutrition these figures rise to between 4,4 and 43,1% (Bengoa, 1970). The nutritional status of children admitted to the Paediatric Hospital wards at a large urban hospital for Blacks in Johannesburg was reviewed by Wagstaff and Geefhuysen (1974). Fifty eight percent of all children between 1 month and 10 years admitted to the medical ward were malnourished. Of these, 50% were severely malnourished. The mortality of those with malnutrition (20%) was twice that of the other children and gastroenteritis was a significant contributory factor. This high incidence of malnutrition, albeit in a selected hospital population, is a serious indictment of nutritional standards in this community.

The association between malnutrition and infection has been recognised throughout history. In recent years this interaction has received considerable attention both in epidemiological surveys and in attempts to elucidate the mechanisms by which malnutrition predisposes to infection. This subject has been widely reviewed (Clausen, 1934; Scrimshaw, Taylor and Gordon, 1959; Hodges, 1964; Scrimshaw, Taylor and Gordon, 1968; Lowenstein, 1970; Gordon and Scrimshaw, 1970; WHO Memoranda, 1972; Gontzea, 1974; Faulk, 1974) and two clear interactions emerge from the evidence. Firstly, judged on an epidemiological basis, infection frequently precipitates a state of severe malnutrition in children

suffering from subclinical forms of the disease; and, secondly, many forms of nutritional deprivation alter the resistance of the host to infection.

The objective of the work I report in this thesis was to examine the effects of one form of severe malnutrition in children - Kwashiorkor - on cellular immune mechanisms. The ultimate goal is to achieve a better understanding of the effects of nutritional disease on the immune response, a more satisfactory basis for clinical care, and a rational basis for prevention.

In the first chapter I have reviewed the literature dealing with the question of whether or not children with severe malnutrition,and kwashiorkor in particular, have a compromised immune defence system.

The second chapter deals with the measures taken to standardise the lymphocyte transformation method for the *in vitro* investigation of cellular immunity in patients with kwashiorkor.

Studies in such children have shown that cellular immune defence mechanisms constitute one important immunological function which is impaired (Smythe, Schonland, Brereton-Stiles, Coovadia, Grace, Loening, Mafoyane, Parent and Vos, 1971; Geefhuysen, Rosen, Katz, Ipp and Metz, 1971; Sellmeyer, Bhettay, Truswell, Meyers and Hansen, 1972). *In vitro* lymphocyte transformation assays have become a standard investigational procedure for examining functions of immunocompetent cells. In order to apply these techniques it was necessary to establish reliable methods that were rigorously standardised.

Details of the study design are outlined in Chapter 3 and the results obtained are analysed and summarised.

A previously unrecognised phenomenon was observed in the course of these experiments: that serum from patients with kwashiorkor was deficient

in its ability to support lymphocte transformation of both autologous and allogeneic lymphocytes. The results of the experiments undertaken in an attempt to define this serum factor in biological and biochemical terms are presented in Chapter 4.

CHAPTER 1

THE INTERACTION BETWEEN MALNUTRITION AND INFECTION

THE EFFECTS OF MALNUTRITION ON THE INCIDENCE AND SEVERITY OF INFECTIONS AND THEIR COMPLICATIONS

Malnutrition and Bacterial Infection

Common clinical experience suggests that malnourished children suffer more frequently and more severely from infections than their well-nourished counterparts. In their description of kwashiorkor, Trowell, Davies and Dean (1954) reported that these children contract infections more frequently, and that the febrile and peripheral blood leukocyte response was feeble when compared with that of well-nourished children. Serious infections spread more rapidly and necrosis occurred in situations where suppuration would be expected. The increased incidence and severity of intercurrent infections are a major cause of death in these children (Scrimshaw, Taylor and Gordon, 1968). In their extensive review of the interactions between nutrition and infection these authors stressed that not only are malnourished children more susceptible to infections, but infections such as measles and acute diarrhoeal disease, superimposed on a deficient diet, are also a major factor in precipitating the development of frank kwashiorkor.

Wittman, Moodie, Fellingham and Hansen (1967) evaluated the relationship between nutritional status and infection in a longitudinal field study of children from various socio-economic groups. Children from families in the lowest socio-economic group had the poorest nutritional status and developed diarrhoeal disease, respiratory infections and multiple illness more frequently and more severely.

The difference in mortality and infection rates between children from socio-economically disadvantaged communities and children from industrialised countries has been studied by Gontzea (1974). He found that mortality rates were closely linked to the economic level and food availability of a community. The peak incidence of deaths occurred in the post-weaning period of 6 to 30 months of age and those due to diarrhoeal disease were 150 to 500 times greater in children from underprivileged nutritionally-deprived groups. The incidence of, and complications arising from, common childhood infectious diseases such as measles, whooping cough, chicken-pox, and mumps was many times greater in this population group.

Investigation of children admitted to hospital with kwashiorkor has shown that nearly all have some form of infection (Gopalan, 1956). Purtillo and Connor (1975) found that 22 out of 25 autopsy cases had severe infections. These included 4 cases of disseminated varicella, 5 children with extensive staphylococcal infection and 3 patients with anergic miliary tuberculosis. Furthermore most of the cases had multiple infections.

Smythe and Campbell (1959) cultured the blood of a series of cases admitted to hospital with kwashiorkor. More than half of those children who died had positive blood cultures, whereas only a small proportion of the surviving cases had positive cultures. The most commonly isolated organisms were gram negative intestinal bacteria, Salmonella species and staphylococci. The authors noted that in spite of positive blood cultures the expected clinical signs of septicaemia such as hyperpyrexia and leukocytosis were absent, and likened the response to that of the infected neonate.

Pretorius, Davel and Coetzee (1956) found a high incidence of infection in a series of 205 cases of kwashiorkor. Twenty seven children

had clinical or radiological evidence of non-tuberculous pulmonary disease. Thirty one out of 40 autopsy cases of kwashiorkor were found by Campbell (1956) to have microscopic evidence of pyogenic abcesses or bronchopneumonia in pulmonary tissue.

Smythe (1958) and Moorhead, Moorhead, Allen and Olsen (1974) found evidence of bacterial infection in more than two-thirds of their cases. Smythe showed that by introducing broad spectrum antibiotic cover early in the therapy of patients with kwashiorkor he was able to reduce the mortality from 45% to 15%. Moorhead *et al.* (1974) found evidence for pneumonia in one third, urinary tract infection in one third, and septicaemia in one quarter of their cases.

Phillips and Wharton (1968) showed a similar high incidence of infection in kwashiorkor with most fatalities occurring in the infected group. Six out of seven positive blood cultures were due to gram negative organisms, and pneumonia and urinary tract infections were common. A remarkably high incidence of infections with *Pseudomonas pyocyaneus* was found in the cases studied by Hendricks (1967).

A high incidence of renal pathology attributable to infection has been described in *post mortem* studies by Stirling (1962) and Campbell (1956). Buchanan, Fairburn, Schmaman and Vos (1973), using a supra-pubic urinary aspiration technique, found that 9 out of 30 children with malnutrition had bacteriologically proven urinary tract infections.

The synergistic relationship between malnutrition and tuberculosis is well recognised, and the anomalous situation in which children with malnutrition and tuberculosis respond in an anergic way to delayed hypersensitivity skin testing with tuberculin suggests impaired immunity to tubercle bacilli (Lloyd, 1968; Harland, 1965; Harland and Brown, 1965). Not only are malnourished children anergic in the presence of tuberculosis, but very often the tuberculous infection behaves in a

disseminated or miliary fashion (Purtillo and Connor, 1975). Scrimshaw *et al.* (1968) point out that tuberculosis gives rise to a negative nitrogen balance and anorexia, and thus is able to precipitate overt malnutrition. An analysis of children admitted to hospital with tuberculosis by Freiman and Geefhuysen (1975) showed that three quarters were malnourished and more than a quarter had frank kwashiorkor. However, they pointed out that this was probably also a reflection on the socio-economic status of the population from which these cases were drawn.

Malnutrition and Gastrointestinal and Diarrhoeal Disease

Of all the diseases attributable to infection in children with malnutrition, diarrhoea is the most frequent and has the most serious consequences for the child. More attention has been focused on this aspect than on any other (Trowell et al., 1954; Gopalan, 1956; Smythe, 1958; Scrimshaw, Taylor and Gordon, 1959; Hansen, Truswell and Purves, 1962; Truswell, Hansen, Schendel and Brock, 1963; Wittman and Hansen, 1965; Wittman *et al.*, 1967; Scrimshaw *et al.*, 1968; Lowenstein, 1970; Gontzea, 1974). Diarrhoea, particularly in the post-weaning period, is more frequent, more severe and often chronic in malnourished children. It is also a prime factor in precipitating the development of frank kwashiorkor in the marginally malnourished child. Pathogenic bacterial isolates from the stools of children with severe malnutrition and diarrhoea have in most studies been relatively low (approximately 40%) and the prevalence of positive viral isolates has not differed significantly from that in cases without diarrhoea (Scrimshaw *et al.*, 1968).

Smythe (1958) showed that there was a regular overlapping of the seasonal prevalence of gastroenteritis and kwashiorkor and that the intestinal flora of the malnourished child with diarrhoea was altered. In the study of Wittman *et al.* (1967) the isolation of multiple pathogens

and *Giardia lamblia* from stool specimens, as well as diarrhoeal disease, were much more frequent in the socio-economically underprivileged children. Of the pathogenic bacteria which have been isolated the most frequent organisms have been salmonellae (Pretorius *et al.*, 1956; Phillips and Wharton, 1968; Scrimshaw *et al.*, 1968).

A study by Heyworth and Brown (1975) has brought forward new evidence for the role of bacterial infection as the cause of diarrhoeal disease in malnutrition. They found that in 22 out of 25 consecutive cases of malnutrition more than 10^5 organisms per millilitre were present in fluid aspirates from the jejunum. Seventeen of these cases had a history of chronic diarrhoea and in these children the prominent organisms were *Escherichia coli*, bacteroides and enterococci. In children with acute diarrhoea streptococcal overgrowth was frequently found. Although appropriate antibiotic therapy on the basis of these isolations resulted in a dramatic improvement in diarrhoea, it was also found that decreased colonisation of the jejunum and clinical improvement accompanied refeeding and removal to a sanitary environment.

Malnutrition and Measles Infection

The combination of measles infection and malnutrition is attended by an illness more severe than would be expected, with a tendency to pulmonary dissemination and a fatal outcome.

In a study involving children from high and low socio-economic groups in which the nutritional status was different Hendricks (1967) found that, although the incidence of infection was similar, fatalities only occurred in the disadvantaged nutritionally deprived group. Both Salomon, Mata and Gordon (1968) and Morley (1969) found that measles in malnourished children behaved in an exaggerated fashion and led to a more severe weight loss than any of the other common childhood acute infections. Diarrhoea

and secondary pulmonary infections are common complications in the malnourished child. The disease is frequently seen in children under 1 year of age and the fatality rate attributable to measles infection in malnourished children is between 6-15% (Gordon and Scrimshaw, 1970).

Burnet (1968) observed that in patients whose cellular immune function was depressed as a result of leukaemia or cortisone treatment, measles infection behaved abnormally and was accompanied by fatal giant cell pneumonia. Smythe *et al.* (1971) in an analysis of autopsy cases of malnutrition and measles found that 78% had giant cell pneumonia and that in less than half a rash was present.

In addition to the fulminant course of measles in malnutrition and its effect in promoting further malnutrition, there is now considerable evidence that cell-mediated immune responses are suppressed by measles infection *per se* (Coovadia, Parent, Loening, Wesley, Burgess, Hallet, Brain, Grace, Naidoo, Smythe and Vos, 1974; Sellmeyer, Bhettay, Truswell, Meyers and Hansen, 1972; Whittle, Bradley-Moore, Fleming and Greenwood, 1973). Antibody production and phagocytic cell function may also be affected by measles infection (Wesley, Hallet and Smythe, 1975; Whittle *et al.*, 1973; Anderson, Sher, Joffe, Rabson and Koornhof, 1975).

Malnutrition and Herpes simplex Infection

Fatal disseminated *Herpes simplex* infection in the non-neonatal period is almost invariably associated with severe malnutrition (McKenzie, Hansen and Becker, 1959; McKenzie, 1961; Hansen, 1961; Becker, Naude, Kipps and McKenzie, 1963). In a review of their findings, Kipps, Becker, Wainwright and McKenzie (1967) reported that 68% of the 93 cases they had seen were severely malnourished and that of the remainder all but 6 were underweight for age. In children admitted to hospital for treatment of acute *Herpes* stomatitis Hansen (1961) reported that the majority

had severe malnutrition including all but 2 of the cases with a fatal outcome, and that of these more than half showed evidence of dissemination at autopsy.

An identical pattern of fatal dissemination of *Herpes simplex* in kwashiorkor is reported by Templeton (1970) from East Africa. He was unable to find any cases in well-nourished children.

Malnutrition and Other Infectious Diseases

The incidence of fungal infections in malnutrition is increased. Campbell (1956) found extensive moniliasis of the upper respiratory and gastrointestinal tract in three kwashiorkor cases seen at autopsy. More than 90% of African children with kwashiorkor were found to have *Tinea capitis* by Van Breuseghem (1957) and Schofield, Perkinson and Jeffrey (1963) reported that infection with *Tinea umbricata* correlated with episodes of failure to gain weight in children.

Examination of lung sections at autopsy for *Pneumocystis carinii* showed that this infection was present in 3 of 39 cases of kwashiorkor and in none of the well-nourished controls (Hughes, Price, Sisco, Havrow, Kapatos, Schonland and Smythe, 1973).

Suskind, Olson and Olson (1973) reported a greater than three-fold increase in the detection of hepatitis-associated antigen in malnourished Thai children.

Malaria and malnutrition are often prevalent in the same communities. However, Hendricks (1967) in analysing retrospective data came to the conclusion that severely malnourished children were less prone to the serious consequences of malaria infection, such as cerebral malaria.

THE EFFECTS OF MALNUTRITION ON IMMUNOLOGICAL DEFENCE MECHANISMS

Effects on the Lymphoid System

The description by Vint in 1937 of thymic atrophy in kwashiorkor is the earliest description of depletion of the immune organs as a consequence of malnutrition. More recent studies have confirmed that in severe protein deficiency in children, atrophy of the thymic gland is extensive (Watts, 1969; Mugerwa, 1971; Smythe *et al.*, 1971; Purtillo and Connor, 1975). The average weight of the thymus was in most cases under 10 g. The thymic weight range for normal children is between 15 and 30 g.

In these *post mortem* studies severe infection as a cause of death was a prominent feature. Smythe *et al.* (1971), Mugerwa (1971) and Purtillo and Connor (1975) found in addition, that lymphoid tissue elsewhere in the body shows a reduction in size with depletion of the paracortical areas of the lymph nodes and spleen. Examination of children with kwashiorkor reveals that lymph nodes are seldom present and reduction in tonsillar mass shows a rough correlation with the degree of malnutrition (Smythe *et al.*, 1971; Chandra, 1972; Neuman, Lawlor, Stiehm, Swendseid, Newton, Herbert, Ammann and Jacobs, 1975).

Histological examination of thymic tissue shows chronic atrophy with a marked loss of small lymphocytes. Most of the studies report that germinal centres in the peripheral lymphoid organs are unaffected but Smythe *et al.* (1971) found that these also showed some depletion in a number of cases.

Peripheral blood lymphocyte counts may be low in malnutrition. Chandra (1972) found that 16% of his cases had a peripheral blood lymphocyte count of less than 2 500 mm^3; Rosen, Geefhuysen, Anderson, Joffe and Rabson (1975) found that a similar degree of lymphopenia was present in 7 out of 20 infected kwashiorkor patients. Neuman *et al.* (1975) found levels of below 1 000 mm^3 in 9% of their cases, but Bhaskaram and Reddy

(1974) and Ferguson, Lawlor, Neuman, Oh and Stiehm (1974) found no differences in the total lymphocyte counts of controls and malnourished patients. Smythe *et al.* (1971) found that the total lymphocyte count correlated to some degree with survival. One out of 63 kwashiorkor patients surviving had counts below 2 500 mm^3, whereas 13 of 49 cases which died had counts below 2 500 mm^3.

The differences in the findings in these surveys may be a reflection of the severity, duration and type of malnutrition present. Reduction in the total lymphocyte count in kwashiorkor may become apparent only when severe and prolonged atrophy of the central and peripheral lymphoid organs has reached a critical degree.

The kinetics of peripheral lymphocyte movement as a response to inflammation in kwashiorkor has been examined in a study from Peru by Freyre, Chabes, Poemape and Chabes (1973) using the Rebuck skin window technique. They demonstrated a failure of migration of mononuclear cells to the site of inflammation in kwashiorkor but not in marasmus. In an *in vivo* study of lymphocytes in the jejunal epithelium, Schwartz, Chitiyo and Wolfsdorf (1974) were unable to detect any difference in the intra-epithelial lymphocyte count of jejunal biopsies done on kwashiorkor and marasmic kwashiorkor infants before and after nutritional recovery.

Effects on Humoral Immunity

Studies of humoral immune function in malnourished children, although extensive, have failed to provide an unequivocal answer to the question of whether or not these children have defective antibody production. Initial studies concluded, on the grounds that total immunoglobulin levels in kwashiorkor and other forms of childhood malnutrition were in most cases normal or raised, that humoral antibody production was unaffected (Watson and Freeseman, 1970; McFarlane, Reddy, Adcock, Adeshina, Cooke

and Akene, 1970; El-Gholmy, Helmy, Hashish, Aly and El-Gamal, 1970; McFarlane, Reddy, Cooke, Longe, Onabamiro and Houba, 1970; Geefhuysen, Rosen, Katz, Ipp and Metz, 1971; Rosen, Geefhuysen and Ipp, 1971; Smythe *et al.*, 1971; Coovadia *et al.*, 1974; Parent, Loening, Coovadia and Smythe, 1974; Neuman *et al.*, 1975). The effects of infection on immunoglobulin levels are particularly difficult to evaluate, as most of these children have some evidence of infection. Keet and Thom (1969) found that there was no difference in IgG and IgM levels between similarly infected controls and kwashiorkor patients, but that IgA levels were much higher in the kwashiorkor patients. Alvarado and Luthringer (1971) similarly found raised IgA levels in their kwashiorkor patients which fell with nutritional feeding and they commented that many of these children had severe diarrhoea.

Chandra (1972) divided his cases into infected and uninfected groups. Those without infection, as judged by clinical and bacteriological criteria, had lower levels of IgG and IgA than the infected patients. Neuman *et al.* (1975) found that, as a group, severely malnourished patients had higher levels of IgG, IgM and IgA than moderately malnourished patients. Geefhuysen *et al.* (1971) were unable to detect any correlation between immunoglobulin levels, the degree of malnutrition and the presence or absence of infection.

In a study from Egypt, Aref, Badr-el-din, Hassan and Araby (1970) found that kwashiorkor children under 1 year of age had very low levels of IgA and IgM. Between 1-2 years of age the IgG level was raised, the IgM level low and the IgA level very variable. Follow-up of these patients showed a persistently low level of IgM. This report is difficult to reconcile with the pattern of raised levels of IgG, IgM and IgA which have been found in Africa, India, Central and South America and in Egypt (El-Gholmy *et al.*, 1970) and may be due to the sampling of a selective

population, as some of their patients are reported as having kwashiorkor at 3 months of age.

Immunoglobulin levels in healthy individuals from a West African village (Rowe, McGregor, Smith, Hall and Williams, 1968) are considerably higher than in European, Scandinavian and North American populations (Stiehm and Fudenberg, 1966; Allansmith, McClellan, Butterworth and Maloney, 1968).

This survey by Rowe *et al.* showed that adult levels of serum IgG were reached at an earlier age (5 years) and that IgM levels rose very rapidly in the first 3 months of life. A relatively high level of IgD was also present in these children. The reason for the higher levels of serum immunoglobulins in African communities is still uncertain, although increased antigenic exposure is a popular explanation. The only reported study of the measurement of IgE levels in malnutrition in Africa by Neuman *et al.* (1975) showed that severely malnourished children had raised IgE levels. Well-nourished African control cases also had raised levels when compared with age-matched controls from the United States. By dividing their African patients into those with and without parasitic infestation they were able to show that this factor correlated to a large extent with the raised levels.

Measurement of secretory IgA in nasal washings from malnourished Thai children was undertaken by Sirisinha, Suskind, Edelman, Asvapaka and Olson (1975). Using carefully controlled experimental techniques they found that secretory IgA was produced by both the malnourished and control children but that the relative concentration of secretory IgA was lower in the malnourished children and only approached control values after relatively long follow-up (84 days). At the same time they found that serum levels of IgA were higher in the malnourished children and gradually returned to normal during dietary treatment. They were unable

to find any correlation between serum and secretory IgA levels and no free secretory piece could be detected in serum.

Cohen and Hansen (1962) measured turnover rates of isotopically labelled albumin and gamma globulin in kwashiorkor patients on a restricted diet and, after recovery, on a full diet. They found that recovery was associated with a greatly increased synthesis of albumin but that, using this indirect technique, gamma globulin synthesis showed only a minimal increase. They also found that gamma globulin synthesis was much greater in infected kwashiorkor patients. Their interpretation of this study was that, whereas kwashiorkor was accompanied by a marked slowing down of albumin synthesis, gamma globulin and therefore antibody production was not affected.

The measurement of specific antibody responses to immunisation with antigens is the second aspect of humoral immune function in children with malnutrition which has been widely explored. However, the interpretation of the results is complicated, not only by the degree of malnutrition and the presence or absence of infection, as in the case of immunoglobulin levels, but also by the fact that antibody responses require a period of time to appear. In most cases the children are given a high protein diet during this period (Scrimshaw, Taylor and Gordon, 1968). In addition, different results have been achieved with different antigens. Pretorius and De Villiers (1962) measured the titres of H and O antibody following immunisation with typhoid and paratyphoid A + B vaccine. Although a few individual kwashiorkor patients had low antibody responses, there was no overall statistically significant difference between the kwashiorkor and control groups. Reddy and Srikanta (1964), using the same antigen, found that those children who were given 50 g protein per day developed higher titres than those receiving 30 g protein per day. Chandra (1972) also found a slightly lower titre of antibody to *Salmonella typhi* O and H

antigens following primer and booster injections in malnourished children. Fernandez (1960) found no difference between malnourished and well-nourished children when using the same antigen, but these patients were receiving vigorous dietary treatment. Coovadia *et al.* (1970), using the same procedure, found lower antibody responses to both H and O antigens in kwashiorkor children. Budiansky and Da Silva (1957), as cited by Scrimshaw, Taylor and Gordon (1968) detected poor antibody responses to typhoid vaccine in children with severe protein malnutrition who were maintained on a poor diet throughout the experimental period. Suskind, Sirisinha, Vithavasai, Edelman, Dapmrongsar, Charupatana and Olson (1976) found lower responses to typhoid antigen when comparing children before and after nutritional refeeding.

Response to other antigens has been variable. Neuman *et al.* (1975), using pneumococcal polysaccharide and keyhole limpet haemocyanin, found no differences in the antibody response of severely malnourished, moderately malnourished and control children. A high protein diet was given to their patients while under study. Antibody responses to yellow fever vaccine and live attenuated polio virus in undernourished children were measured by Brown and Katz (1965). Response to polio immunisation was normal. Those children with antibody present prior to immunisation did not excrete virus, and those with negative titres became colonised, excreted the virus in their stools and developed positive antibody titres. Response to yellow fever vaccine was, however, deficient in the kwashiorkor patients. A group of undernourished children was also immunised with smallpox vaccine and all developed a normal vesicular skin reaction. No details of dietary therapy are given in this study. El-Molla, El-Ghoroury, Hussein, Badr-el-Din, Hassan, Aref and El-Araby (1973) measured the responses of kwashiorkor, marasmic and normal children to cholera antigen given three times, at weekly intervals. They found no differences in

response, using a vibriocidal assay, but lower levels of agglutinating antibody using the cholera antigen. Jose, Welch and Doherty (1970) measured the antibody response to influenza virus in aboriginal children with a high erythrocyte sedimentation rate (ESR) and evidence of chronic growth retardation. Compared with aboriginal children with a low ESR and with healthy Caucasian children, the antibody responses were lower. The degree of antibody response was inversely proportional to the serum levels of IgG and IgM, and treatment of these children with penicillin and antiparasitic drugs caused an improvement in antibody responses. The authors felt that antigenic competition was a factor in the pathogenesis of diminished antibody responses to immunisation.

Mathews, Whittingham, MacKay and Malcolm (1972) and Mathews, MacKay, Tucker and Malcolm (1974) showed that by giving an augmented protein diet for 7 months following immunisation, antibody response in rural New Guinea schoolchildren to flagellin was higher than in children not receiving any dietary supplementation.

Chandra (1975a) immunised 20 malnourished children with live attenuated measles and polio virus and measured antibody responses in the serum and in the salivary secretions. Normal serum levels of antibody were produced but specific secretory IgA antibody was detected less often, its appearance was delayed and maximum levels obtained were lower than in control children.

Effects on Cell-Mediated Immunity

Cell-mediated immunity in malnutrition has been investigated in three ways: *in vivo* by the delayed hypersensitivity test and *in vitro* by measuring lymphocyte transformation in response to phytohaemagglutinin, and by quantitating the number of T lymphocytes in peripheral blood.

Delayed hypersensitivity reactions to tuberculin following BCG vacci-

nation could not be elicited in a large percentage of malnourished children studied by Harland and Brown (1965) and Harland (1965), even when a higher dose of tuberculin was used for testing. In 51 cases of malnutrition who had evidence of active tuberculosis Lloyd (1968) found that only 11 had a positive Heaf test, and even when 100 tuberculin units were used in testing nearly half still gave a negative response. In a group of 103 well-nourished children with comparable tubercular infection all had positive responses with the Heaf test.

Chandra (1972) found a lower conversion rate following BCG immunisation in malnourished children using 5 and 50 tuberculin units as the testing antigen. A diminished response to PPD in marasmic children who had received BCG at birth, and also in infected well-nourished controls was found by Schlesinger and Stekel (1974). Abassey, Badr-el-Din, Hassan, Aref, Hammad, El-Araby,Badr-el-Din, Soliman and Hussein (1974) vaccinated normal, kwashiorkor, marasmic-kwashiorkor and marasmic children with BCG. All developed a papule at the site of vaccination 3 weeks later. All the normal children, but only 10% of the malnourished children showed a positive delayed hypersensitivity response to 5 tuberculin units two months after immunisation. By 18 months, however, these negative reactors all became positive on repeated testing. They showed a strong correlation between body weight, serum albumin and the ability to respond to test antigen, and all but 2 children, who had achieved a serum albumin of 2,7 g/100 ml and 70% of their expected weight for age, failed to respond to the test antigen. From this evidence the authors surmised that sensitisation took place but that recall was dependent on the nutritional status.

Delayed hypersensitivity skin reactions to other antigens have also shown a decreased response in malnourished children. Geefhuysen, Rosen, Katz, Ipp and Metz (1971) found a significantly diminished response in

kwashiorkor patients to testing with *Candida albicans* and diphtheria toxoid antigens. Most of the cases which gave negative initial responses to *Candida* became positive 3 weeks after dietary therapy. Neuman *et al.* (1975) obtained similar results using *Candida* and streptokinase-streptodornase. They also found a decreased response to phytohaemagglutinin injected intradermally in severely malnourished children. Jose *et al.* (1970) reported a decreased response to *Candida* in aboriginal children with a high ESR and evidence of chronic undernutrition. Chandra (1972) found a similar pattern using *Candida*, streptococcal and *Trycophytin* antigens in malnourished children.

A decreased ability to respond to di-nitro-chloro-benzine (DNCB) following sensitisation has been reported by a number of workers (Smythe *et al.*, 1971; Coovadia *et al.*, 1974; Parent *et al.*, 1974; Chandra, 1972; Bang, Mahalanabis, Mukherjee and Bang, 1975) in kwashiorkor, and in marasmus (Schlesinger and Stekel, 1974). Edelman, Suskind, Olson and Sirisinha (1973) used DNCB to sensitise a group of patients with protein calorie malnutrition. They measured the inflammatory response 2 days later at the site of application and subsequently challenged them with a test dose of DNCB 70 days later to measure the delayed hypersensitivity response. *Candida* skin tests were also performed at these times. They found a defective inflammatory response to DNCB in their patients when this was applied prior to the initiation of feeding and that subsequent challenge of negative inflammatory responders was also negative for delayed hypersensitivity reactions. Negative responders to *Candida* became positive when rechallenged after dietary therapy. The authors reasoned that there was a defect in sensitisation and the inflammatory response in these children, as well as a defect in the efferent delayed hypersensitivity response. This finding is in conflict with the experiments conducted by Abassay *et al.* (1974) who found that sensitisation was

possible with BCG vaccine but that the efferent response was suppressed. The major difference between these studies was that in the one case BCG was given intradermally and in the other DNCB applied topically, and this may be the explanation for the different patterns of sensitisation observed.

Abassay, Badr-el-Din, Hassan, Aref, Hammad, El-Araby and Badr-el-Din (1974) in a second paper investigated the immediate hypersensitivity response in their patients. They found a significantly decreased reaction to common allergens with the scratch test and were unable to achieve passive cutaneous transfer reactions in kwashiorkor patients, who were, however, able to respond with a normal wheal and flare to histamine. Smythe *et al.* (1971) and Coovadia *et al.* (1974) were able to elicit DNCB delayed hypersensitivity responses in their kwashiorkor patients after nutritional recovery, but no mention is made of the initial inflammatory response to DNCB. Schlesinger and Stekel (1974), in their study on marasmic children, found that initial negative responders following DNCB sensitisation remained negative up to 26 months later.

The defective migration of lymphocytes to an inflammatory area as demonstrated by the Rebuck skin window technique (Freyre *et al.*, 1973) may partially explain the defective inflammatory response to DNCB and the inability to achieve sensitisation and to elicit subsequent delayed hypersensitivity responses.

In vitro techniques for the measurement of lymphocyte responses to mitogens have provided a new means of quantitating the effects of nutrition on cellular immunity. Following the original description of impaired cellular immunity in kwashiorkor by Smythe *et al.* (1971) there have been numerous reports confirming and substantiating their findings. One of the problems in comparing the results of these studies is that

the *in vitro* techniques vary widely. Differences in mitogen dosage, cell concentration, serum supplementation, culture conditions and radioisotopic labelling procedures are numerous. Results may be obtained by morphological examination for blast transformation, or by radioactive precursor incorporation, and in many cases these are expressed as indices.

Smythe *et al.* (1971) cultured peripheral blood lymphocytes in autologous plasma and assessed the response by counting the number of blastoid and mitotic cells present in stained smears after 72 hours. There was a significant reduction in the percentage of cells with a blastoid or mitotic appearance in kwashiorkor patients. Geefhuysen *et al.* (1971) and Coovadia *et al.* (1974) using a similar technique achieved the same results. They also found that following dietary therapy transformation returned to normal levels. Bhaskaram and Reddy (1974) found a decreased number of blast cells in kwashiorkor and marasmic patients. Although the value for marasmic children returned to normal with treatment, the kwashiorkor patients showed a persistent defect.

Schonland, Shanley, Loening, Parent and Coovadia (1972) reported that they found a strong correlation between the reduction in number of blast cells seen and raised levels of unbound serum cortisol. Schlesinger and Stekel (1974), in contrast to Bhaskaram and Reddy (1974), found no abnormality in marasmic children but details of the serum supplementation in their cultures were not given.

Using radioactive thymidine incorporation Sellmeyer *et al.* (1972) found depressed lymphocyte transformation rates in kwashiorkor patients. Cultures were set up in foetal calf serum and results expressed as a stimulation index. These authors also found suppression of lymphocyte responses in patients who had measles or gastroenteritis, but not in those with pneumonia.

Chandra (1972) achieved similar results but no details of the serum used in his cultures were given. Both Ferguson *et al.* (1974) and Moore, Heyworth and Brown (1974) found no differences between controls and malnourished patients by measuring radioactive thymidine incorporation of PHA-stimulated lymphocytes. The serum used in the cultures of Ferguson *et al.* was not stated and Moore *et al.* used foetal calf serum and 2 different doses of PHA. Neuman *et al.* (1975), giving stimulation indexes, reported a reduction in lymphocyte transformation in malnourished patients. No details of the serum used are given and some of their cases had recently had measles. Burgess, Vos, Coovadia, Smythe, Parent and Loening (1974) reported a discrepancy between the results obtained using morphological and radioisotopic precursor incorporation. The cultures from which smears were made for blast cell counting were set up in autologous serum and all 7 kwashiorkor patients had depressed numbers of blast cells. In contrast, cultures of radioactive thymidine incorporation were set up in AB serum and only 2 out of 8 kwashiorkor patients had abnormal results.

Quantitation of peripheral blood T and B lymphocytes has been another approach to the immunological investigation of children with severe malnutrition. Chandra (1974), Bhaskaram and Reddy (1974), Ferguson *et al.* (1974) and Bang *et al.* (1975) have used the spontaneous sheep red cell rosette technique to quantitate the number of T cells in peripheral blood lymphocytes of malnourished children. All of them demonstrated a decreased percentage of rosette forming cells. Chandra reported that following nutritional therapy the percentage rose to normal, but Bhaskaram and Reddy found depressed levels one month after dietary therapy was started in their patients. Chandra and Bang *et al.* measured the number of lymphoid cells forming rosettes with complement-coated red cells and found no differences in the numbers of these

when compared with control patients. Coovadia *et al.* (1974) measured the numbers of immunoglobulin-bearing peripheral blood lymphocytes with an immunofluorescent technique and found no difference between control values and those of patients with kwashiorkor.

Effects on Phagocytic Cell Function

The early observations that children with kwashiorkor had a depressed leukocyte response to infection (Trowell, Davies and Dean, 1954; Scrimshaw, Gordon and Taylor, 1968) stimulated investigation into polymorph leukocyte activity and function in malnutrition. Tejada, Argueta, Sanchez and Albertazzi (1964) reported that they could detect no deficit in phagocytic ability, using an index of the ability to digest *Staphylococcus aureus*, in children with kwashiorkor. Arbeter, Echeverri, Franco, Munson, Velez and Vitale (1971) were unable to find any deficiency in the bactericidal activity of phagocytic cells from children and adults with severe malnutrition. Seth and Chandra (1972) demonstrated an increased number of viable bacteria in control polymorphs incubated with malnourished patients' serum. Using control plasma they also demonstrated a deficiency in the bactericidal capacity of the patients' phagocytic cells, but the range of activity was widely distributed between individual patients and overlapped with control values. Kendall and Nolan (1972) reported decreased bactericidal activity in malnourished children.

In a detailed investigation of phagocytic function in children with malnutrition Selveraj and Bhat (1972a, 1972b) found a decrease in killing of *E. coli* organisms and decreased glycolytic and hexose monophosphate shunt activity during phagocytosis in their patients. The resting levels for these parameters were, however, high. They also found that autologous serum was not responsible for these defects and that improvement occurred

with nutritional rehabilitation. Leukocyte enzymes levels were measured in kwashiorkor and control polymorphonuclear cells. No differences were detected in glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase and acid phosphatase activity. Granule bound NADPH oxidase activity was both lower and did not respond with an increase, following phagocytic stimulation. There was also defective release of acid phosphatase from the white cell granules into the supernatant during phagocytosis. Nutritional therapy resulted in a return to normal activity. The authors felt that the reduced NADPH oxidase levels would account for the depressed hexose monophosphate shunt activity and bactericidal activity of the patients' phagocytic cells.

In a similar study Douglas and Schopfer (1974) found defective killing of *E. coli* and staphylococcus organisms by the phagocytic cells of kwashiorkor patients. However, they were unable to detect any decrease in hexose-monophosphate shunt activity. Phagocytosis of red cells and latex particles was normal and no deficiency in opsonic activity of the patients' serum was found.

The investigations of Rosen, Geefhuysen, Anderson, Joffe and Rabson (1975) into leukocyte function in kwashiorkor revealed that the presence of infection can affect the findings. Both infected kwashiorkor patients and control children demonstrated low bactericidal and chemotactic activity of their neutrophils, when compared with uninfected kwashiorkor patients and controls.

The nitro blue tetrazolium reduction test had been widely used as a test of polymorph function in kwashiorkor and malnourished children. No differences were detected between controls and malnourished children in several studies (Altay, Say, Dogramaci and Bingo, 1972; Rosen *et al.*, 1975), while others showed deficient reduction in malnourished patients (Shousha and Kamel, 1972; De Buse, 1974).

Various enzyme and substrate levels in the leukocytes of malnourished children have been measured by Yoshida, Metcoff, Frenk and De la Pena (1967) and Yoshida, Metcoff and Frenk (1968). Reduced levels of pyruvate, lactate, oxaloacetate and adenosine triphosphate were found in conjunction with a reduction in pyruvate kinase activity. Leukocyte lysozyme levels have also been found to be low in kwashiorkor patients (Mohanram, Reddy and Mishra, 1974).

As with *in vitro* lymphocyte studies, the techniques used for the investigations of phagocytic cell function are subject to many variations, such as the purity and numbers of cell populations, the type and ratio of organisms or particles used, and the presence or absence of infection in the patient.

Effects on the Complement System

Children with kwashiorkor have, as a characteristic and diagnostic feature, serum albumin levels that are usually below 3,0 g/100 ml and often below 2,0 g/100 ml. Low levels of beta globulin accompany this fall in albumin, whereas the alpha and gamma globulin levels are often normal. One of the beta globulins, complement component C3 has been found to be low in children with malnutrition by a number of investigators (Chandra, 1972; Coovadia *et al.*, 1974; Neuman *et al.*, 1975; Chandra, 1975b) and the haemolytic activity of complement (CH_{50}) was also low (Smythe *et al.*, 1971; Chandra, 1975b). Chandra reported that levels of C3 and CH_{50} were lower in those children who had obvious infections in addition to malnutrition, and that anticomplementary activity and raised levels of immunoconglutinin were often present. Smythe *et al.* reported increased adherence of C4 to red cells.

A detailed investigation of the various complement components in kwashiorkor and marasmus, using specific antisera and immunodiffusion

techniques was undertaken by Sirisinha, Suskind, Edelman, Charupatawa and Olson (1973). On admission, before treatment was initiated, C1q, C1s, C3, C5, C6, C8, C9 and C3PA serum levels were low, but C4 levels were not significantly different from controls. By day 29 there was a pronounced increase in all components, often with some overshooting of the control values, except in the case of C9. All levels, but especially C1q, C6 and C8 were lower in kwashiorkor than in marasmic patients. When dietary protein content between days 7-29 was restricted to 1 g per day, there was no rise in the levels of the subnormal components. Caloric restriction had a minimal effect on the return to normal values. Children who had severe infections had lower levels than those without infections.

On the basis of these findings and in particular the normal levels of C4, the authors felt that decreased synthesis, rather than increased consumption via C1q and the classical pathway of complement activation, accounted for the low levels of the other complement components. However, the fact that levels were lower in cases with infection suggests that some degree of increased consumption may also be a factor, possibly via the alternate pathway.

Normal levels of C4 with low levels of C3 have also been reported by Neuman *et al.* (1975) and other investigators have similarly found that complement levels rapidly return to normal with adequate dietary supplementation (Coovadia *et al.*; Chandra, 1975b).

Ratnakar and Ramachandraiah (1974) have studied the effects of kwashiorkor serum on the growth of *E. coli in vitro*. Growth rates for this organism were much higher in both fresh and heat-inactivated kwashiorkor serum, although heat-inactivation increased the rate of growth in both normal and malnourished serum. By adding 4 parts of normal serum to 10 parts kwashiorkor serum they could abolish the

increased growth pattern. This paper raises some interesting questions in that, although heat-inactivation abolished some of the bacteriostatic effects of both sera, there are clearly other non-heat-labile factors which inhibit bacterial growth in normal serum.

CONCLUSIONS

i) Undernourished children appear to contract infectious diseases more frequently than their well-nourished counterparts. This is due both to a decreased resistance to infection and to social and economic factors such as overcrowding and poor sanitation.

ii) When children with severe protein-energy malnutrition contract infections, the pattern of disease which evolves is more severe, is subject to complicating factors and carries a greater morbidity and mortality than it does in well-nourished children.

iii) There is considerable evidence to indicate that protein-energy malnutrition exerts a deleterious effect on cell-mediated immune responses. Atrophy of the thymus gland and depletion of the thymic dependent areas in peripheral lymphoid organs is well established. Functional responses as measured *in vivo* by delayed hypersensitivity responses and *in vitro* by lymphocyte responses to mitogens, show a decreased level of activity which is compatible with cellular immune deficiency syndromes. The fact that organisms such as rubella virus, herpes simplex virus, tubercle bacilli and gram negative bacteria give rise to fulminant disease in both undernourished children and in patients with congenital or drug-induced cellular immune deficiency, provides further support for the concept of depressed cellular immunity in severe protein-energy malnutrition.

iv) Other aspects of immune function are affected by undernutrition. The production of specific antibodies may be defective in the child with kwashiorkor. Reports in which children have been studied whilst not receiving protein supplementation indicate that this is so, but insufficient evidence is available, or is ethically likely to become available, in this regard. Serum complement component levels are low in children with protein-energy malnutrition and this appears to be in most part due to decreased synthesis of protein. Phagocytic cell function may not be normal in these children and although there are inconsistencies in the various reports, the overall picture is one of some impairment. The inflammatory response as measured by delayed hypersensitivity, immediate hypersensitivity and the Rebuck skin window techniques, exhibits abnormal behaviour.

v) There is thus compelling reason to believe that the immune status of the malnourished child is deficient, and that this deficiency contributes to mortality and morbidity.

CHAPTER 2

THE STANDARDISATION OF METHODS FOR MEASURING LYMPHOCYTE TRANSFORMATION IN VITRO

INTRODUCTION

During the initial stages of this work, very variable lymphocyte mitogenic responses were observed. A study of the factors which might be responsible for the poor reproducibility was, therefore, undertaken and procedures were standardised to minimise these factors.

The experimental method selected as a starting point for this investigation was the one described in detail by DuBois, Huismans, Schellekens and Eijsvoogel (1973) who had conducted a similar investigation. This method was restricted in its usefulness for investigating serum factors effecting lymphocyte transformation, because of the relatively large volumes of serum that were required. A second assay system was developed on a microscale which utilised a tenth of the volume of serum, but still retained the discrimination, sensitivity and reproducibility of the original method. It had, in addition to the lesser amounts of cells and serum required, the advantage of a semi-automated harvesting system which improved the reproducibility and cut down on the time taken to complete this stage.

The first section of this chapter reports a review of the literature and the results of the experiments which were done to establish and standardise the measurement of lymphocyte transformation responses *in vitro*. In the second section a description of the methods used in this thesis for measuring lymphocyte responsiveness to mitogens and in the mixed lymphocyte culture is detailed. These methods were derived from the results of the experiments reported in the first section of this chapter.

TECHNICAL FACTORS AND CULTURE CONDITIONS AFFECTING THE LYMPHOCYTE TRANSFORMATION TEST

The *in vitro* lymphocyte transformation assay involves the measurement (usually indirect) of the blastogenic response of lymphocytes to antigens and mitogens of variable potency and composition. Furthermore, conditions of culture that are used may vary widely, so that the comparison of results is often not possible, even when full details of the experimental technique are furnished. In many reports these details are lacking. Considerable attention has been directed towards improving the quantitative value of this test by standardising the materials and culture conditions employed. This section is devoted to a review of published procedures and reports the experiments that I have done to standardise and control experimental conditions.

Anticoagulation of Venous Blood

The two commonly used means of anticoagulating fresh whole blood are heparinisation and defibrination. Lymphocytes can also be obtained from blood anticoagulated with EDTA and acid-citrate-dextrose, but in these cases the chelated Ca^{++} ions must be replaced before use in the lymphocyte transformation test. When heparin (without preservative 20 u/ml) is used for anticoagulating blood, platelets separate with the lymphocytes in the separation procedures and may cause clumping of the lymphocytes (Holt, 1966; Ling and Kay, 1975). This clumping of cells makes accurate counting and dispensing of cells in suspension difficult, and gives rise to poor reproducibility when replicate tubes are assayed. When blood is defibrinated with glass beads the platelets are removed with the fibrin clot and an

evenly dispersed single cell suspension is readily obtained. For these reasons defibrination of blood was the standard anticoagulant procedure employed in this study.

Storage of Anticoagulated Blood

Although lymphocyte survival in anticoagulated blood is reported to be excellent for at least 24 hours (Pauly Sokal and Han, 1973), most investigators have preferred to use freshly obtained blood (Ling and Kay, 1975).

Lymphocyte cultures obtained from freshly defibrinated blood were compared with cultures established from the same blood sample which had been stored for 24 hours at $4^{o}C$. The amount of $[^{14}C]$ - thymidine incorporated by these replicate cultures is shown in Table 2.1. The response to phytohaemagglutinin of the lymphocytes obtained from blood stored at $4^{o}C$ for 24 hours in this experiment shows a slightly enhanced response, which cannot be explained by spontaneous transformation as the unstimulated cultures show no increase.

In the investigations reported in this study lymphocytes were obtained only from freshly obtained blood samples with a maximum delay between bleeding and separation of 6 hours.

Separation Techniques

Many different techniques have been developed for the isolation of lymphocytes from whole blood (Denman, 1973). Most methods employ the use of red cell aggregating agents such as Dextran, methyl cellulose or Ficoll (Böyum, 1968a). The combination of red cell aggregation and density centrifugation as described by Böyum (1968b) has proved to be highly reproducible, rapid and efficient and is thus widely used for obtaining lymphocytes for short term tissue culture.

TABLE 2.1

PHA-INDUCED LYMPHOCYTE TRANSFORMATION OF LYMPHOCYTES OBTAINED FROM FRESH BLOOD AND FROM THE SAME BLOOD WHICH HAD BEEN STORED AT $4^{o}C$ FOR 24 HOURS.

	Time after bleeding	
	0 hours	24 hours
PHA-stimulated	7377*	7858
	7611	9357
	8139	9637
	8012	9973
	7528	9997
Unstimulated	43	20
	115	47
	255	107
	53	58
	253	26

*Disintegrations per minute of $[^{14}C]$ -thymidine incorporated by 3×10^{5} lymphocytes in replicate cultures.

Böyum's method involves the centrifugation of diluted blood which has been layered over a solution of Ficoll and sodium metrizoate (Isopaque) with a density of 1,077 g/ml. The cellular components of whole blood are separated into two fractions; lymphocytes and monocytes (and platelets when heparinised blood is used) are retarded above the separation fluid while granulocytes and aggregated red cells fall through to the bottom of the tube.

The basic principles and method have remained unchanged but minor modifications have been introduced in recent years (Thorsby and Bratlie, 1970; Böyum, 1974).

Factors affecting the yield, purity and viability of lymphocytes obtained with the Ficoll-Isopaque technique include the diluting of whole blood, the density of the separation fluid, the size and diameter of the centrifugation vessel, the gravitational force exerted and the temperature at which centrifugation is performed (Böyum, 1968b; Thorsby and Bratlie, 1970; Böyum, 1974).

Du Bois *et al.* (1973) reported that PHA-induced blastogenesis of lymphocytes separated on a Ficoll-Isopaque gradient were superior to those obtained by Leukopak filtration and Dextran sedimentation, and felt that this was due to the absence of large numbers of contaminating red cells which were present in the latter preparations.

Yachnin and Raymond (1975) using the same methods found no difference in response. Lymphocytes purified on glass bead columns have been successfully used in the lymphocyte transformation test (Simons, Fowler and Fitzgerald, 1969).

Lymphocytes in unfractionated whole blood respond to phytohaemagglutinin stimulation (Park and Good, 1972; Pauly, Sokal and Han, 1973). This method has the advantages of avoiding separation pro-

cedures and requires only small volumes of blood, but it is limited in its usefulness by the difficulty in standardising the number of lymphocytes in the cultures, and by the inability to differentiate between lymphocyte and humoral factors affecting the response.

The adjustment of Ficoll-Isopaque to a density of 1,077 g/ml is important as minor variations affect the yield and purity. Initially a hydrometer was used, but this proved cumbersome and relatively inaccurate. The technique as described by Gorczynski, Miller and Phillips (1970), in which the refractive index is used to measure the density of unknown solutions was adapted for measuring the density of the Ficoll-Isopaque solution (Fig. 2.1) and this has proved to be efficient and accurate. The average yield of lymphocytes as calculated from the total and differential counts on whole blood has always been between 50-90%. Lymphocyte suspensions of lower yield were discarded as there may have been selective loss of lymphocyte subpopulations. The viability of the lymphocyte populations after isolation was determined by incubating the cells with Trypan blue stain (see Appendix). The number of cells excluding Trypan blue was in all cases greater than 95% and any preparation with a lower percentage of viable cells was discarded.

The effect of other cells on the response of lymphocytes to phytohaemagglutinin has been investigated by a number of authors. Schellekens and Eijsvoogel (1968) and Fitzgerald (1971) found that the presence of granulocytes did not affect the lymphocyte transformation response to phytohaemagglutinin provided the number of lymphocytes was kept constant. However, Ling and Kay (1975) point out that extracts of granulocytes can actively break down thymidine, affecting the results if radioactive thymidine incorporation is used

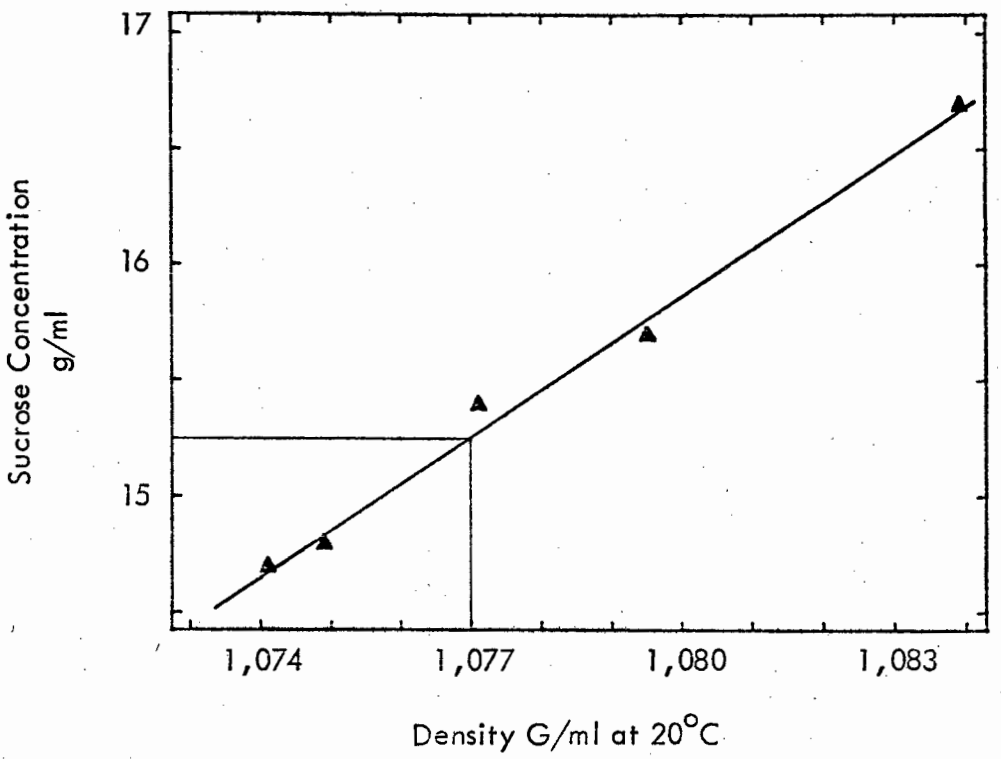


FIGURE 2.1 Calculation of the specific gravity of Ficoll-Isopaque solutions.

This graph shows the linear relationship between the sucrose content of Ficoll-Isopaque solutions measured with a refractometer and the specific gravity of the solutions measured gravimetrically.

Note that a specific gravity of 1,077 corresponds to a sucrose content of 15,3 g/100 ml.

as a measure of tranformation. Contaminating cells may also use up limiting nutrient material or impede cell to cell contact. Du Bois *et al.* (1973) found that addition of erythrocytes to pure lymphocyte cultures enhanced PHA responses but depressed antigen stimulation.

Examination of stained smears from lymphocyte suspensions prepared by Ficoll-Isopaque sedimentation showed that up to 10-15% of the mononuclear cells had morphological features that resembled monocytes. Practically, however, it was extremely difficult to differentiate between large lymphocytes and monocytes using conventional staining techniques. Monocytes can be readily differentiated from lymphocytes by identifying the esterase activity in the former. Using an esterase stain (see Appendix) the number of monocytes in Ficoll-Isopaque separated mononuclear cell preparations was identified. In 6 experiments the number of monocytes ranged from 12-17%.

Monocytes have been reported to be essential for normal lymphocyte responses in the mixed lymphocyte culture and to antigens, but not for their response to mitogens (Lohrmann, Novikovs and Graw, 1974; Hersch and Harris, 1968). To test this hypothesis monocytes were removed from whole blood by the carbonyl iron method. Five millilitre of whole blood was incubated with a knife tip of carbonyl iron (G.A.F. Corporation, New York) at 37^{o}C and gently agitated. The iron-containing monocytes (and other phagocytic cells) were removed by decanting the blood whilst a strong magnet was held to the side of the tube. The lymphocytes were then isolated in the conventional manner by centrifugation on a Ficoll-Isopaque gradient. Examination of both Leishman and esterase-stained smears showed that this form of treatment effectively removes most of the monocytes (Table 2.2). When the lymphocyte transformation response to PHA of the monocyte-

TABLE 2.2

PERCENTAGE OF BLOOD CELL TYPES IN VARIOUS PREPARATIONS AS DETERMINED BY COUNTING 200 CELLS ON SMEARS STAINED WITH LEISHMANS AND ESTERASE STAINS, AND THEIR RESPONSE TO PHA STIMULATION.

	Unfractionated Blood	Ficoll Isopaque Isolated Mono-nuclear Cells	Ficoll Isopaque Isolated Mono-nuclear Cells Following Carbonyl Iron Treatment
Polymorphs	44	2	-
Lymphocytes	51	85	99
Monocytes	2	13	1
Eosinophils	3	-	-
$[^{14}C]$ -thymidine incorporation by 3×10^5 lymphocytes following PHA stimulation (replicate cultures)		22515 23724 24851 25612	25185 23324 25329 22377

poor preparation (1% monocytes) was compared with the preparations in which monocytes were not removed, no differences were found in replicate cultures (Table 2.2).

It is interesting to note that there is a relative enrichment of monocytes in the mononuclear cell suspensions prepared by the Ficoll-Isopaque centrifugation process.

In the experiments reported here there were always less than 5% contaminating granulocyte cells, but monocytes were not removed from the cell suspension.

Culture Tubes and Plates

Initially lymphocyte transformation cultures were established in sterile 12 x 75 mm capped tubes (Falcon, cat. no. 2054). However, considerable variation in replicate cultures was present and when a new batch of tubes was obtained, counts were very much lower and the replicate variation increased. It was then discovered that Bain and Lowenstein (1965) had reported the presence of toxic substances in the same Falcon tubes and that these substances varied from batch to batch. These toxic substances were held to be responsible for lower counts and greater variation.

Du Bois *et al.* (1973) had similarly reported that Falcon 10 x 75 mm polystyrene tubes (cat. no. 2038) had substances which were toxic for lymphocyte cultures even after one year of storage. They found that Nunc polystyrene tubes (cat. no. 1090) gave better and more reproducible results. Experimental evidence for the superiority of Nunc tubes was obtained by culturing lymphocytes simultaneously in these tubes and in Falcon Plastic tubes, and in another experiment by comparing the Nunc tubes with sterile 10 x 90 mm round-bottomed glass tubes. The results of these two experiments are shown in

TABLE 2.3

$[^{14}C]$ -THYMIDINE INCORPORATION IN LYMPHOCYTE CULTURES WITHOUT, OR STIMULATED WITH, PHA, IN FALCON PLASTIC (2054) AND NUNC (1090) PLASTIC POLYSTYRENE TUBES.

	PHA-stimulated		Unstimulated	
	Falcon	Nunc	Falcon	Nunc
$[^{14}C]$ -thymidine incorporation (replicate tubes)	19585	36294	70	105
	18222	30644	78	127
	21301	36739	132	132
	22827	35299	51	103
	27316	36542	78	96
Coefficient of variation	16,1	7,3	36,7	14,2

TABLE 2.4

$[^{14}C]$ -THYMIDINE INCORPORATION IN LYMPHOCYTE CULTURES WITHOUT, OR STIMULATED WITH, PHA, IN NUNC POLYSTYRENE TUBES (1090) AND STERILE 10 x 90 mm ROUND-BOTTOMED GLASS TUBES.

	PHA-stimulated		Unstimulated	
	Glass	Nunc	Glass	Nunc
$[^{14}C]$ -thymidine	37260	35387	122	348
incorporation	24084	33993	156	202
(replicate tubes)	29049	37653	119	256
Coefficient of variation	22,1	5,2	15,2	27,5

Tables 2.3 and 2.4. In both cases the stimulated cultures showed higher counts and less variation between replicates in the Nunc plastic tubes. No real differences in the unstimulated cultures were observed, although the replicates were better in the Nunc than in the Falcon polystyrene tubes.

The lymphocyte transformation experiments on the patients with kwashiorkor and on the control specimens were all done in Nunc polystyrene tubes (cat. no. 1090). The investigation of kwashiorkor serum was done in a microculture system in which polystyrene microtitre plates were used. Du Bois *et al.* (1974) explored the suitability of various types of microtitre plates in mixed lymphocyte cultures and in PHA-stimulated cultures. They found no differences between Linbro and Cooke round-bottomed plates in PHA-stimulated cultures and found the Cooke round-bottomed plates superior to the Linbro flat-bottomed and the Falcon flat-bottomed plates. On the basis of their experience Cooke U-bottomed plates (cat. no. 220 M - 24 AR and cover M 220 - 42 - 29 AR) have been used in all the microlymphocyte culture experiments.

Culture Medium and Buffers

Short term lymphocyte cultures have been established in a wide variety of tissue culture media with various buffer systems. The choice of medium and buffer is often determined by what is available and in use in a particular laboratory. Schellekens and Eijsvoogel (1968) found no difference in the results obtained with Eagles Minimum Essential Medium (Eagles MEM) or Medium 199. They also found comparable results when cultures were buffered with sodium bicarbonate in a 5% CO_2 atmosphere, or with Tris buffer in closed tubes. Yachnin and Raymond (1975) found that RPMI 1640 culture medium gave the best

TABLE 2.5

$[^{14}C]$ -THYMIDINE INCORPORATION BY 3 x 10^5 PHA-STIMULATED LYMPHOCYTES IN DIFFERENT MEDIA

	Eagles MEM with 0,025 M Tris Buffer	RPMI 1640 with Bicarbonate Buffer and 5% CO_2
Disintegrations per minute (replicate tubes)	36294	35475
	30644	31970
	36739	30329
	35299	30244
	36542	30709

stimulation results, but that Eagles MEM (spinner salts), Medium 199 and Eagles MEM (Earles salts) were not significantly lower. NCTC 109 was not as good as any of these media.

Preliminary experiments showed that in lymphocyte cultures in tubes there was little difference when cultures were established in either Eagles MEM with Tris buffer, or in RPMI 1640 with bicarbonate buffer. The tris-buffered culture tubes were closed and the bicarbonate-buffered tubes exposed to 5% CO_2 (Table 2.5).

A further experiment was carried out in which three variables were considered, Eagles MEM versus RPMI media, Hepes Bicarbonate buffer versus Tris buffer and control AB serum versus inhibitory kwashiorkor serum. The Eagles MEM and RPMI 1640 media were standard commercial media (Gibco). The buffers used were 0,025 M Tris in the one case and 0,015 M Hepes with 0,025 mM sodium bicarbonate buffer in the other. The Hepes Bicarbonate-buffered cultures were incubated, loosely capped, in a 5% carbon dioxide incubator and the Tris-buffered cultures in tightly capped tubes. The serum used in these experiments was either 20% normal pooled AB serum or 20% kwashiorkor serum which was known to be poorly supportive for PHA stimulation.

Cultures were established in either Nunc tubes or Cooke round-bottomed microplates in the usual way. The Cooke round-bottomed plates with lids were tightly wrapped in Jiffy sandwich wrap which prevents evaporation at 37^{o}C but still permits diffusion of CO_2 in the Hepes Bicarbonate-buffered cultures.

The results of the cultures in tubes are shown in Table 2.6.a and a three-way analysis of variance is shown in Table 2.6.b. The results of cultures in microtitre plates and three-way analysis of variance are shown in Tables 2.7.a and 2.7.b.

TABLE 2.6.a

[^{14}C] -THYMIDINE INCORPORATION BY PHA-STIMULATED LYMPHOCYTES IN NUNC TUBES WITH VARIOUS MEDIA, BUFFERS AND SERA

x serum	AB serum				Kwashiorkor serum			
y medium	RPMI 1640		Eagles MEM		RPMI 1640		Eagles MEM	
z buffer	Tris	Hepes Bicarb	Tris	Hepes Bicarb	Tris	Hepes Bicarb	Tris	Hepes Bicarb
d.p.m.*	35188	41733	37370	43743	12465	17956	13401	28159
(replicate	36078	39884	29067	43396	13762	19786	8226	26146
tubes)	28970	37809	34828	45871	13113	18871	10814	27153

*d.p.m. = disintegrations per minute.

TABLE 2.6.b

THREE-WAY ANALYSIS OF VARIANCE OF DATA IN TABLE 2.6.a

	Source of Variation	Degree of Freedom	Sum of Squares	Mean Squares	F	P
x	serum	1	$2{,}482 \times 10^9$	$2{,}482 \times 10^9$	416,2	<0,01
y	medium	1	$4{,}417 \times 10^7$	$4{,}417 \times 10^7$	7,4	<0,05
z	buffer	1	$5{,}726 \times 10^8$	$5{,}726 \times 10^8$	96,0	<0,01
x.y	serum v. med.	1	$4{,}626 \times 10^5$	$4{,}626 \times 10^5$	0,1	N/S*
y.z	med. v. buffer	1	$8{,}177 \times 10^7$	$8{,}177 \times 10^7$	13,7	<0,01
z.x	serum v. buffer	1	$9{,}825 \times 10^6$	$9{,}825 \times 10^6$	1,6	N/S
xyz		1	$1{,}534 \times 10^7$	$1{,}534 \times 10^7$	2,6	N/S
remainder		16	$9{,}542 \times 10^7$	$5{,}964 \times 10^6$		

* N/S = non significant

TABLE 2.7.a

$[^{14}C]$ -THYMIDINE INCORPORATION BY PHA-STIMULATED LYMPHOCYTES IN COOKE ROUND-BOTTOMED MICROTITRE PLATES WITH VARIOUS MEDIA, BUFFERS AND SERA.

x serum	AB serum				Kwashiorkor serum			
y medium	RPMI 1640		Eagles MEM		RPMI 1640		Eagles MEM	
z buffer	Tris	Hepes Bicarb	Tris	Hepes Bicarb	Tris	Hepes Bicarb	Tris	Hepes Bicarb
d.p.m.*	9561	20521	10184	18889	3653	15218	881	11156
(replicate	12296	23291	8584	17235	3613	15107	1307	15367
wells)	12426	22894	10394	11305	5070	16865	1180	8336

*d.p.m. = disintegrations per minute.

TABLE 2.7.b

THREE-WAY ANALYSIS OF VARIANCE OF DATA IN TABLE 2.7.a

	Source of Variation	Degree of Freedom	Sum of Squares	Mean Squares	F	P
x	serum	1	$2{,}655 \times 10^8$	$2{,}655 \times 10^8$	59,0	<0,01
y	medium	1	$8{,}701 \times 10^7$	$8{,}701 \times 10^7$	19,4	<0,01
z	buffer	1	$5{,}707 \times 10^8$	$5{,}707 \times 10^8$	126,9	<0,01
x.y	serum v. med.	1	$4{,}007 \times 10^5$	$4{,}007 \times 10^5$	0,1	N/S*
y.z	med. v. buffer	1	$1{,}279 \times 10^7$	$1{,}279 \times 10^7$	2,8	N/S
z.x	serum v. buffer	1	$1{,}021 \times 10^7$	$1{,}021 \times 10^7$	2,3	N/S
xyz		1	$4{,}853 \times 10^6$	$4{,}853 \times 10^6$	1,1	N/S
remainder		16	$7{,}194 \times 10^7$	$4{,}496 \times 10^6$		

*N/S = non significant

As expected from preliminary studies, in both systems there were significant differences between AB serum and kwashiorkor serum. There was also in both systems a significant difference between the two buffer systems used. Hepes Bicarbonate buffer with 5% CO_2 gave higher results in both tube and microplate cultures. In the microplate system RPMI 1640 medium was clearly superior to Eagles MEM in that higher tranformation was present. However, in tube cultures Eagles MEM medium gave higher transformation rates than RPMI 1640. This may be due to a significant interaction between Eagles MEM and Hepes Bicarbonate buffer which gave high transformation results (Table 2.6.b).

Of particular importance to this study was the fact that no significant serum x medium or serum x buffer interactions were observed. In other words, neither medium nor buffer influenced the effect of kwashiorkor serum. The differences detected between AB and kwashiorkor serum were equal or slightly more pronounced in the combination of Eagles MEM with Tris buffer than in any of the other systems. This is shown graphically in Figure 2.2. For this reason it was decided to conduct the experiments in Eagles MEM with Tris buffer as the standard culture medium. This culture medium is economical, easy to standardise, does not require a CO_2 incubator and provides the essential requirements when supplemented with serum for lymphocyte transformation.

FIGURE 2.2

FIGURE 2.2 The effects of kwashiorkor serum on lymphocyte blastogenic responses in various culture media and buffers.

Cultures of PHA-stimulated lymphocytes were established in Nunc tubes (panel a) and microplate wells (panel b). Media were supplemented with either AB or kwashiorkor serum as shown at the bottom of the panels. In each case pairs of points connected by lines represent the mean $[^{14}C]$ -thymidine incorporated by the same lymphocytes cultured in triplicate in various combinations of media and buffers and the two different sera.

Media-buffer combinations were as follows:

Eagle's MEM and Hepes Bicarbonate Buffer: ▲——▲
RPMI 1640 and Hepes Bicarbonate Buffer : ▲- - -▲
Eagle's MEM and Tris Buffer : △——△
RPMI 1640 and Tris Buffer : △- - -△

Note that (i) in both systems (tube and microplate cultures) there are significant differences between AB and kwashiorkor serum;

(ii) in both systems cultures buffered with Hepes/bicarbonate in 5% CO_2 (solid triangles) gave higher results;

(iii) in the microplate system (panel b) RPMI 1640 medium (dotted lines) gave higher results than Eagle's MEM whereas the converse was true in tube cultures (panel a);

(iv) no significant serum x medium or serum x buffer interactions were present;

(v) the differences between AB and kwashiorkor serum were slightly more pronounced in the combination of Eagle's MEM and Tris buffer.

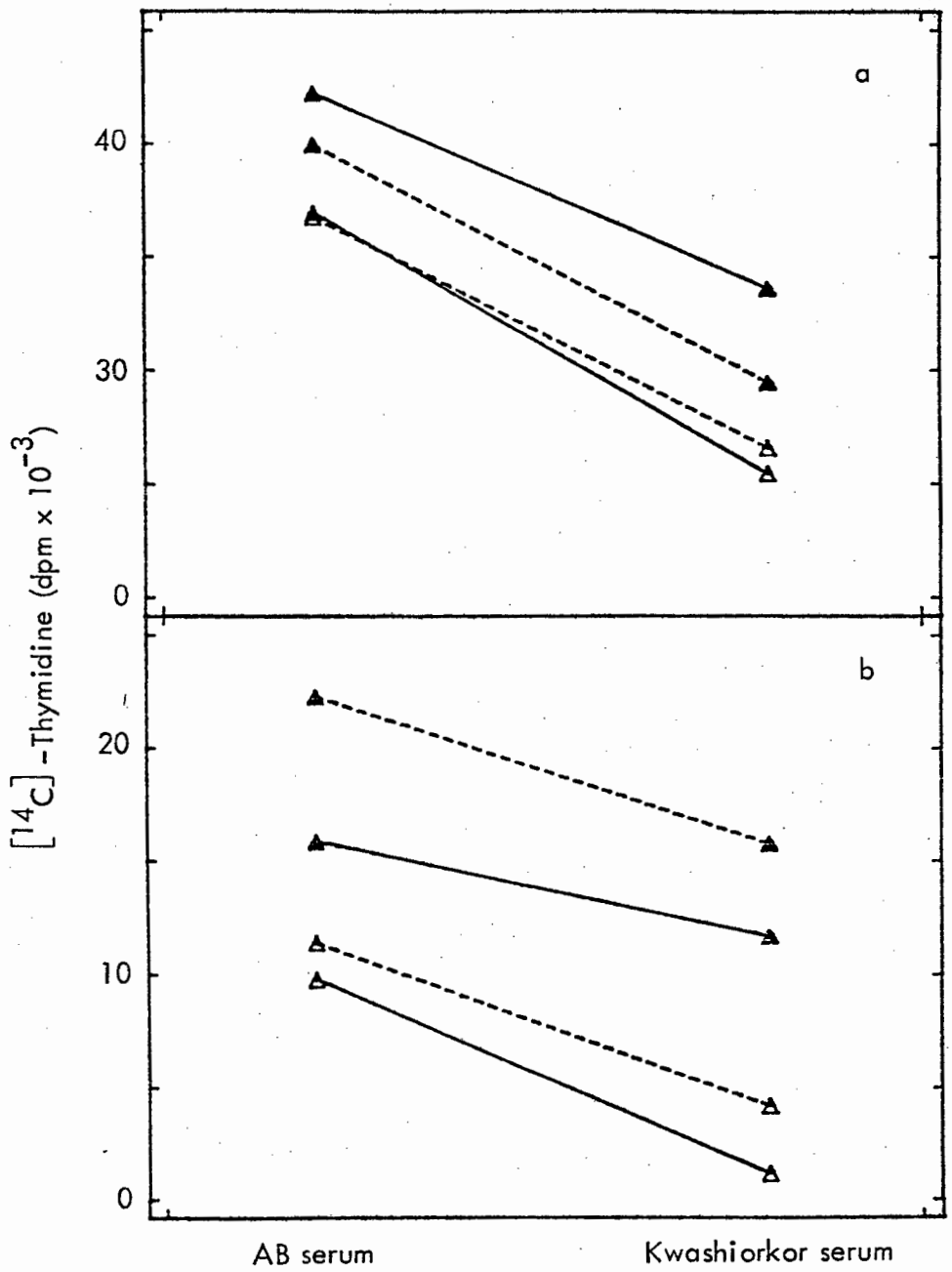


FIGURE 2.2 The effects of kwashiorkor serum on lymphocyte blastogenic responses in various culture media and buffers.

Cell Concentration

The effect of cell concentration on the lymphocyte response to mitogenic stimuli is influenced by the volume of culture fluid, the shape of the vessel, the nutrient medium and the buffers used. Schellekens and Eijsvoogel (1968) and Yamamura (1973) found a linear relationship between cell number and uptake of radioactive thymidine. In systems where there is a restriction on the volume of medium, serum and buffers, linearity is lost when nutrient factors become limiting. Du Bois *et al.* (1973) and Stewart, Cramer and Steward (1975) found that in 1 ml culture volumes, 1 x 10^5 lymphocytes gave optimal results. The results of an experiment in which various numbers of lymphocytes were made up in a 1 ml volume of Eagles MEM medium with 20% AB serum and 1 µg of PHA/ml are shown in Fig. 2.3.a. Thymidine incorporation increased linearly with cell concentration up to 3 x 10^5 cells/ml after which uptake per cell diminished rapidly. At cell concentrations above 5 x 10^5/ml no further increase in $[^{14}C]$ -thymidine with cell number was observed. All tube cultures therefore were established with 3 x 10^5 cells in 1 ml of culture medium.

A similar experiment was done to determine the effects of varying the cell concentrations in 0,2 ml volume microplate cultures containing 12,5% serum and 0,5 µg PHA/well. The results depicted in Fig. 2.3.b show a linear increase in $[^{14}C]$ -thymidine incorporation with cell number up to a concentration of 10^6 cells/ml after which linearity is lost. I therefore chose 2 x 10^5 cells in 0,2 ml final culture volume as optimal for this system.

FIGURE 2.3

FIGURE 2.3 The effect of lymphocyte concentration on PHA-induced blastogenic responses.

Phytohaemagglutinin stimulation of cultures containing different concentrations of lymphocytes were established in Nunc tubes (panel a) and microtitre plates (panel b). Each point represents the mean value of $[^{14}C]$ -thymidine incorporated during standard culture conditions by triplicate cultures.

Note that in the tube cultures (panel a) $[^{14}C]$ -thymidine incorporation increased linearly with cell concentration up to 3×10^5/ml and that above 5×10^5 lymphocytes/ml no further increase was observed. In microplate cultures (panel b) a linear increase in $[^{14}C]$ -thymidine incorporation with cell number was observed up to a concentration of 10^6/ml, after which linearity was lost.

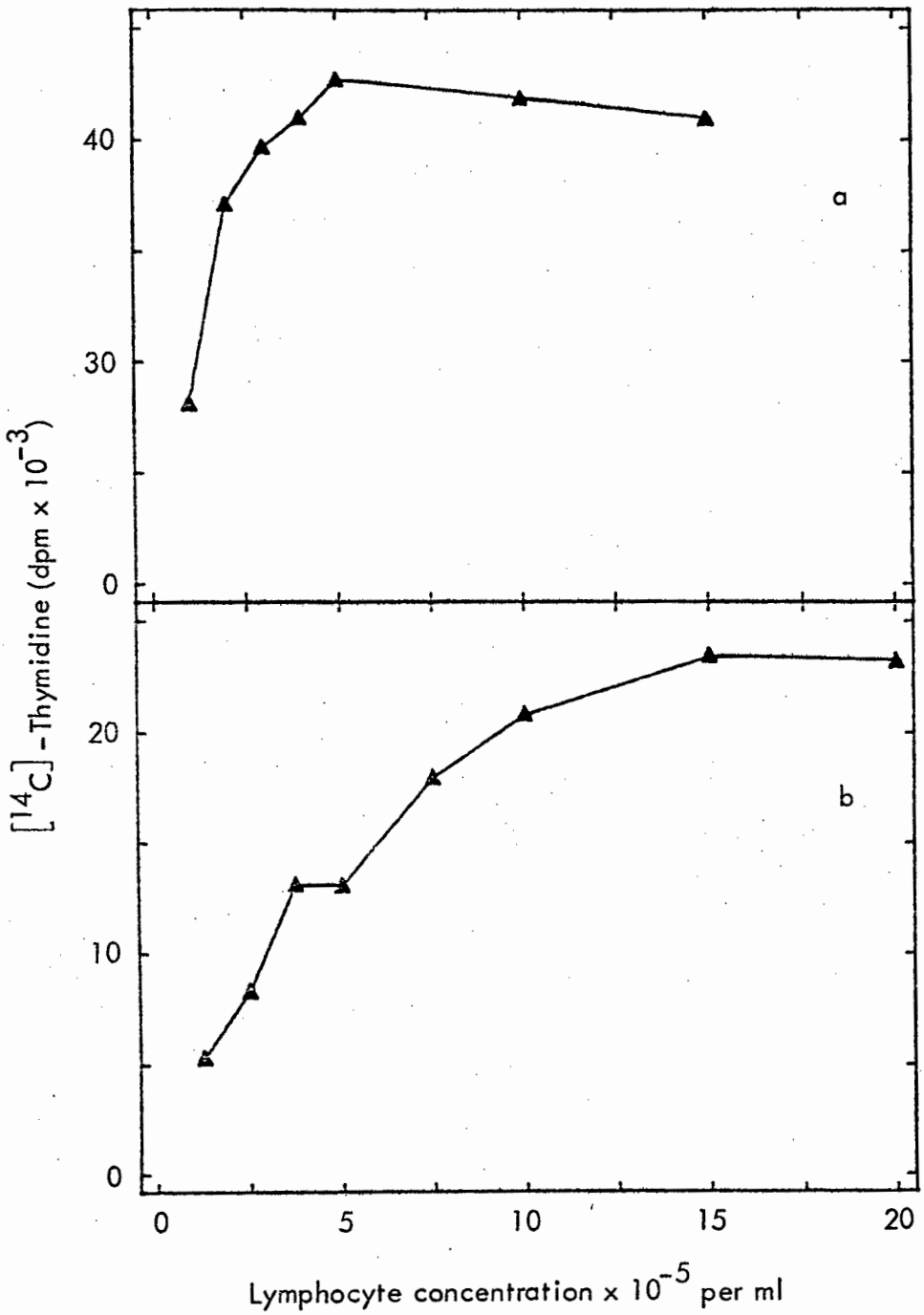


FIGURE 2.3 The effect of lymphocyte concentration on PHA-induced blastogenic responses.

Source of Serum

Confidence in lymphocyte transformation results can only be achieved if the culture medium, conditions of incubation and reagents can be standardised. The standardisation of medium, test tubes and culture conditions is a relatively simple matter. The most difficult component to standardise is the serum. Because serum is an ill-defined and variable reagent and because lymphocyte responses have been shown to vary widely according to the source of serum in the incubation mixture, much attention has been directed towards attempting to define an ideal serum source, or an alternative source for the essential nutrients present in serum. In spite of this effort no wholly acceptable solution has been reached. Many authors have reported that sera from different donors may show marked differences in their ability to support lymphocyte transformation (Holt, 1966; McIntyre and Cole, 1969; Ling and Kay, 1975). In addition there is an increasing list of human conditions in which the patients' serum has been reported to depress lymphocyte transformation. These conditions include malignant disease, gastrointestinal disorders, renal disease, infections and miscellaneous disorders including pregnancy, multiple sclerosis, *Ataxia telangiectasia*, allergy and rheumatoid arthritis (Nelson and Gatti, 1976).

The most satisfactory approach to obtaining a standard source of serum has been to obtain a large pool of serum from healthy adults and to store the serum frozen in small aliquots which are thawed just prior to use. Schellekens and Eijsvoogel (1968) found little difference in lymphocyte responses when cultured in either autologous serum, pooled blood group AB Rh +ve serum (AB serum) or foetal calf serum (FCS). However they found, as did others (Johnson and Russell,1965; Ling and Kay, 1975) that FCS gave high background values in unstimulated cultures. By heat-inactivating serum at 56^{o}C all complement activity is abolished

without affecting the supportive ability (Yachnin and Raymond, 1975).

The need for a pooled human serum source has also been stressed by Fudenberg *et al.* (1971) and Ling and Kay (1975). Du Bois *et al.* (1973) reported that AB serum stored for up to 1 month at 4°C gave as good results as fresh AB or autologous serum.

Another approach has been to culture lymphocytes in medium without serum to which various substances have been added (Coulson and Chalmers, 1967; Vischer, 1972; Katz-Heber, Peck and Click, 1973). In human lymphocyte cultures serum-free medium has not been successful (Yachnin and Raymond, 1975).

In order to obtain a suitable source of standard serum for use in the experiments reported here, a unit of serum (approximately 250 ml) was obtained from each of four young male group AB Rh +ve blood donors who had no detectable cytotoxic antibodies in their serum. The sera were heat-inactivated at 56°C for 30 min and then sterilised by pressure filtration through a 0,45 µ millipore filter.

Lymphocytes were obtained from five healthy blood donors and cultures established with 20% serum from each of the serum donors. Lymphocyte transformation responses were measured by $[^{14}C]$ -thymidine incorporation in triplicate PHA-stimulated and unstimulated cultures and the results are depicted graphically in Fig. 2.4.

These results show that each unit of serum had a different capacity for supporting lymphocyte transformation and also that cells from each lymphocyte donor were responsive to PHA to a different degree. No serum x lymphocyte interactions were observed in that the relative responses of the five cell types were the same in each serum tested. The four units of serum were pooled (± 1 000 ml) and stored frozen in 3 ml volumes at -80°C. Aliquots were thawed just prior to use. One year after this batch of serum had been prepared a new batch was prepared in idential

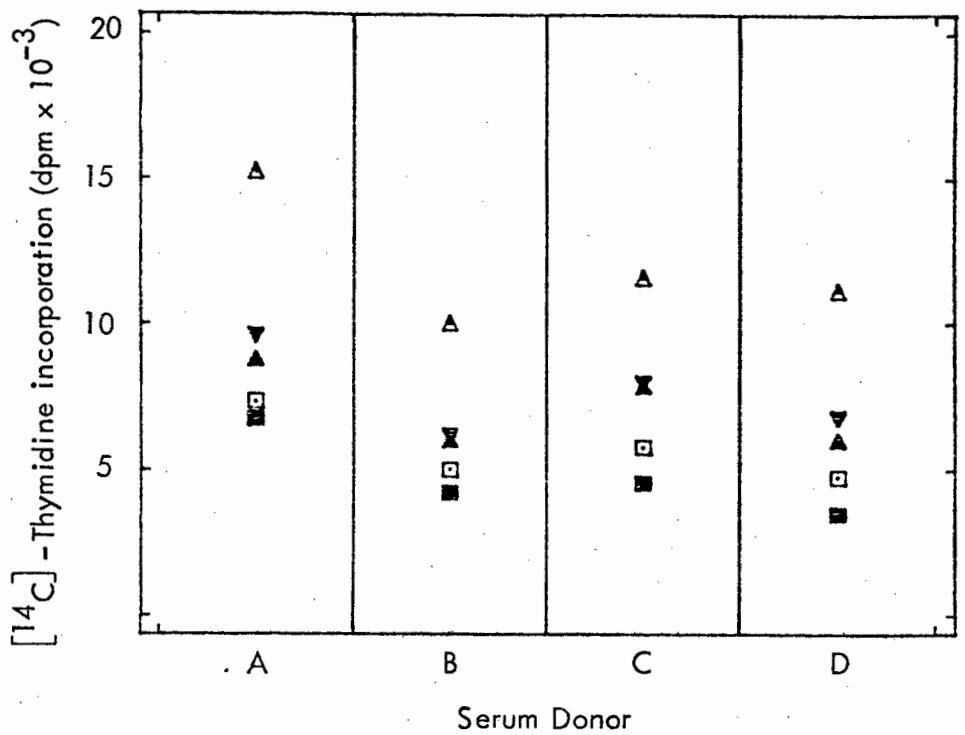


FIGURE 2.4 The effects of sera from different donors on lymphocyte blastogenic responses.

Lymphocytes obtained from 5 healthy adults (each represented by a different symbol) were cultured in the presence of serum obtained from 4 healthy adult males with blood group AB Rh +ve. The plotted symbols depict the mean values of PHA-stimulated $[^{14}C]$ -thymidine incorporation in triplicate lymphocyte cultures containing serum from donors A, B, C and D (shown in separate panels).

Note that each unit of serum had a different capacity for supporting lymphocyte transformation and that cells from each lymphocyte donor were responsive to PHA to a different degree. The relative responses of the 5 cell types were the same in each serum tested.

fashion. Comparison of serum from these two pools showed that the AB serum pool which had been stored frozen at -80°C for 1 year supported lymphocyte transformation responses as well as did the fresh pool.

Serum Concentration

The serum concentration providing optimal support for lymphocyte transformation depends on the other culture conditions, i.e. volume and type of incubating medium, cell concentration, culture tubes and incubating conditions. Serum concentration curves were established in tube cultures (1 ml volume, 3 x 10^5 lymphocytes) and in microtitre plate cultures (0,2 ml volume, 2 x 10^5 lymphocytes). The results of these experiments are shown in Figs. 2.5.a and 2.5.b. In tube cultures the optimal serum concentration was 20% and this finding has been confirmed in repeated experiments, although in some cases the results obtained in 10% serum were nearly as good as those obtained in 20% serum. In most culture systems between 15-20% serum has been found to be optimal (Ling and Kay, 1975).

From Fig. 2.5.b it can be seen that in microplate cultures the serum requirement was no longer limiting above a concentration of 12,5% in the medium. This marginal concentration was chosen because of the need to conserve the limited amounts of serum available from children with kwashiorkor and because it coincided with the concentration recommended by Yachnin and Raymond (1975).

Phytohaemagglutinin Concentration

Phytohaemagglutinin (PHA) from a standard source was used in all experiments (Burroughs Wellcome Purified, cat. no. HA 16) and dose response curves were constructed in order to select an optimal PHA dose for the culture system used in this work. PHA dose response curves at various

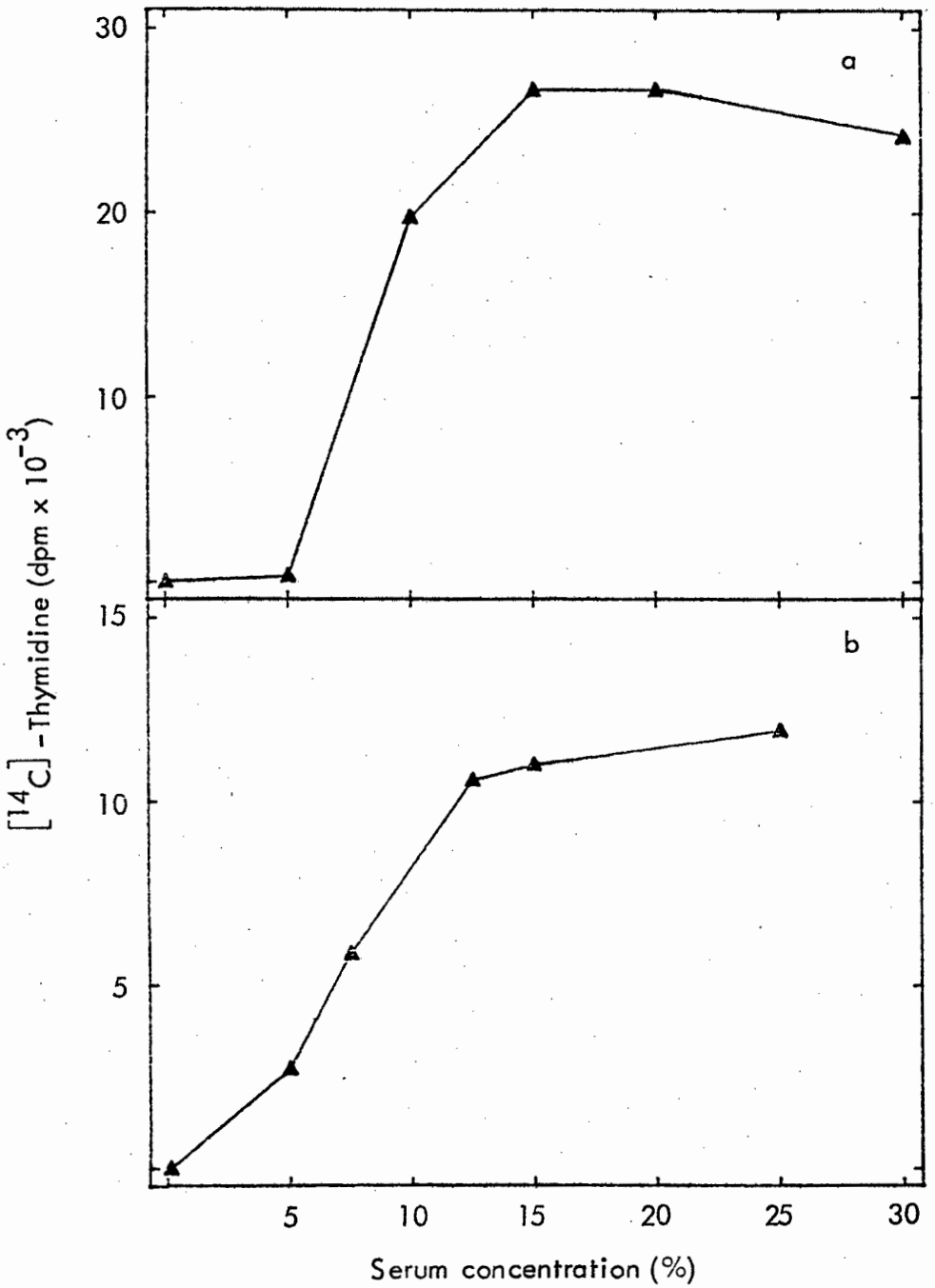


FIGURE 2.5 The effect of serum concentration on lymphocyte blastogenic responses.

Each point on the graph shows the mean value of $[^{14}C]$ -thymidine incorporated by triplicate PHA-stimulated lymphocyte cultures in Nunc tubes (panel a) and microplate wells (panel b) at different serum concentrations.

Note that optimal blastogenic responses were seen at serum concentrations of between 15% and 20% in tube cultures and 12,5% and 15% in microplate cultures.

serum concentrations in tube cultures are shown in Fig. 2.6. The maximal response obtained at 1 µg/ml was identical to that obtained by Yamamura (1973) using similar culture conditions and the same source of PHA. Interaction between serum concentration and PHA concentration has been reported (Forsdyke, 1967). PHA dose curves constructed at different serum concentrations as depicted in Fig. 2.6 showed that, at all serum concentrations, maximal stimulation was achieved between 0,8 - 1,6 µg PHA/ml, and that 20% serum was optimal.

Similar dose-response curves in microtitre plates are presented in Fig. 2.7.b and compared with data from the same experiment for tube cultures (Fig. 2.7.a). As can be seen from the figures, the microplate system required a higher concentration for maximal stimulation (3,0 µg/ml) than did the tube cultures. All experiments reported here utilised 1 µg of Burroughs Wellcome purified PHA per millilitre in tube cultures or 0,5 µg per 200 µl in the case of microplate cultures.

Dilution of PHA for addition to cultures was done each week and solutions were stored at -20°C. Testing of stored dilute solutions showed no loss of activity up to 1 month after dilution (Table 2.8).

Incubation Periods

In PHA-stimulated cultures the rate of DNA synthesis peaks after approximately 72 hours in culture (Michalowski, 1963; Epstein and Stohlman, 1964). Most authors have cultured PHA-stimulated lymphocytes for a total of 3 days with a 4-24 hr pulse of radioactive thymidine at the end of this period (Schellekens and Eijsvoogel, 1968; Du Bois *et al.*, 1973; Penhale, Farmer, MacCuish and Irvine, 1974).

Using a final 24 hr pulse with $[^{14}C]$ -labelled thymidine, PHA-stimulated lymphocyte cultures were harvested at various times. The results (Fig. 2.8.a) show that maximal radioactive thymidine incorporation

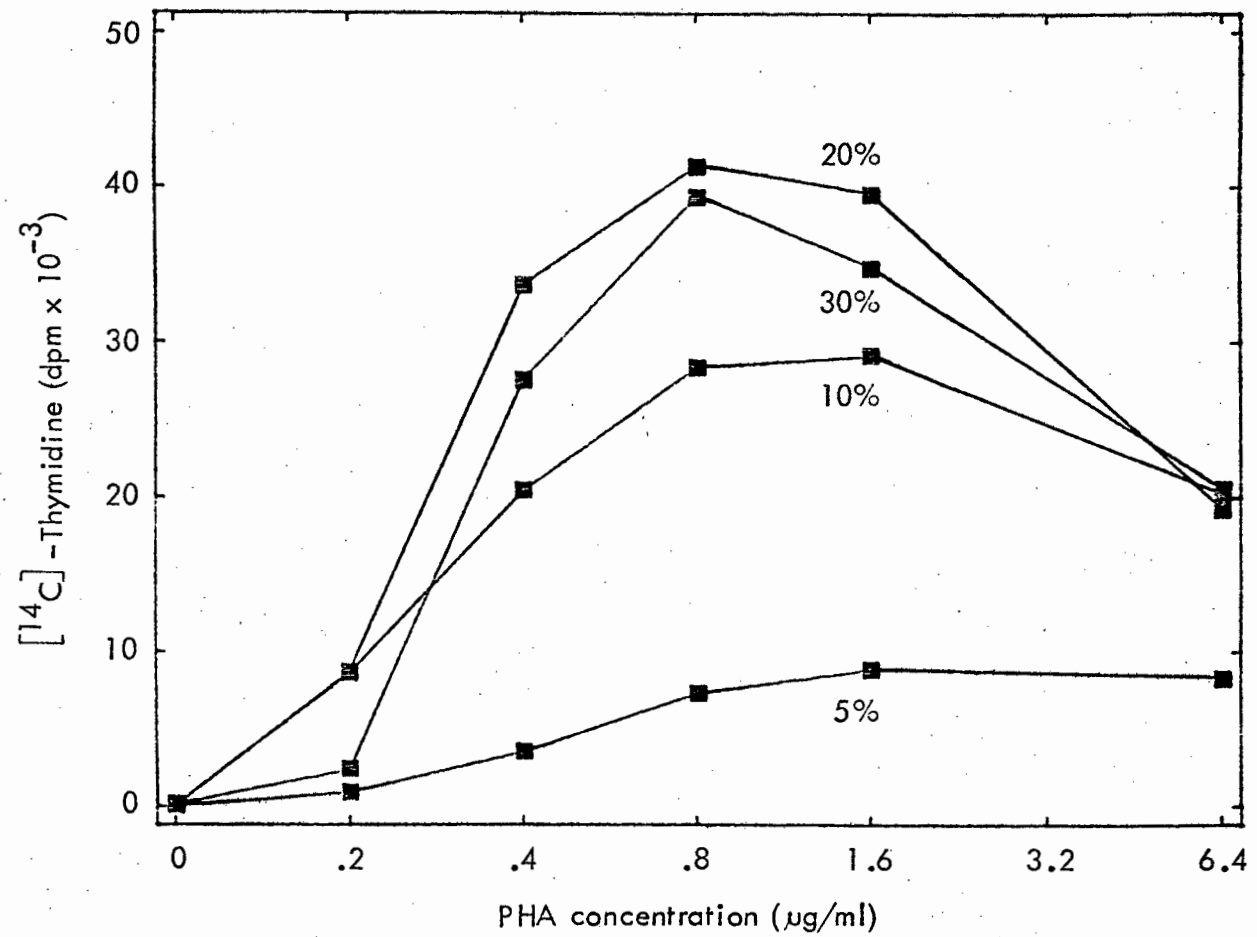


FIGURE 2.6 The effect of phytohaemagglutinin (PHA) concentration on the blastogenic response of lymphocytes cultured in media containing different concentrations of serum.

Lymphocyte cultures in Nunc tubes were stimulated with different concentrations of PHA. The points on each graph represent the mean value of $[^{14}C]$ -thymidine incorporated by triplicate cultures containing 5%, 10%, 20% and 30% normal AB serum.

Note that at all serum concentrations maximal lymphocyte stimulation was achieved at a PHA concentration of between 0,8 and 1,6 µg/ml, and that incorporation of radioactivity was greatest at a 20% serum concentration.

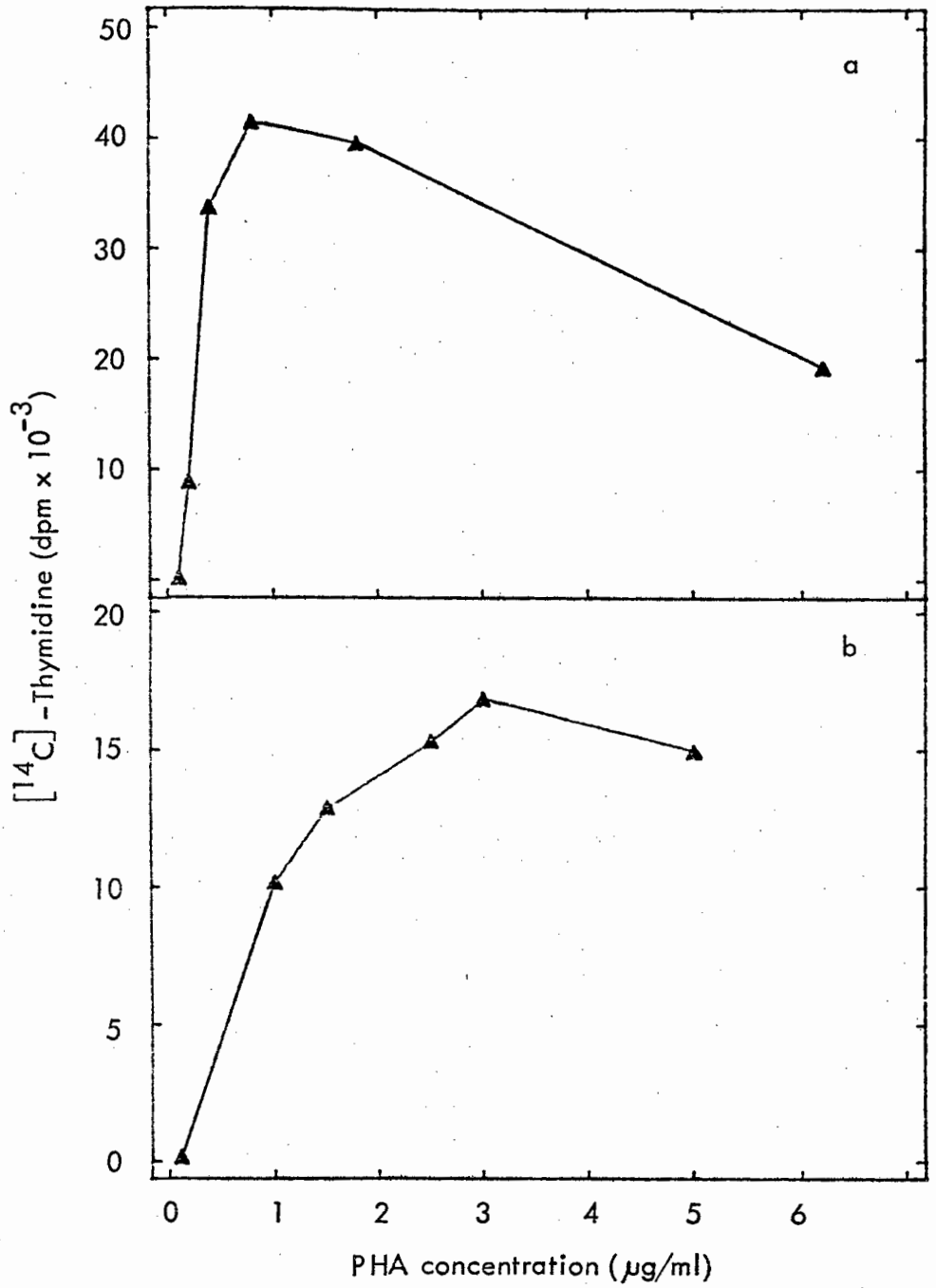


FIGURE 2.7 The effect of phytohaemagglutinin (PHA) concentration on lymphocyte blastogenesis.

Triplicate lymphocyte cultures were established in Nunc tubes (panel a) and in microplate wells (panel b) and stimulated with different concentrations of PHA. The points on each graph represent the mean values of $[^{14}C]$ -thymidine incorporation.
Note that optimal PHA stimulation is achieved at a concentration of 1 µg/ml in tube cultures, and 3 µg/ml in microplate cultures.

TABLE 2.8

LYMPHOCYTE TRANSFORMATION RESPONSES TO 1 μg OF BURROUGHS WELLCOME PURIFIED PHA STORED IN A DILUTE FORM (1 μg/50 μl) FOR VARIOUS PERIODS OF TIME

Duration of storage of dilute PHA	$[^{14}C]$ -Thymidine incorporation
1 day	29756*
14 days	30007
24 days	28608
31 days	30062
35 days	26828

* Mean value of triplicate cultures.

Disintegrations per minute x 10^{-3}.

occurred at 3 days. In a second experiment 0,075 μCi [^{14}C] -thymidine (62 mCi/mMole) was added to triplicate cultures at various time intervals before harvesting all the cultures at a total incubation time of 72 hours. Fig. 2. 9.a shows that maximal incorporation of radioactivity was achieved when [^{14}C] -thymidine had been present in the cultures for 20 hr and that the values obtained at 20 and 24 hr were similar.

Identical results were obtained when the same experiments were conducted with stimulated cultures in microtitre plates (Figs. 2.8.b and 2. 9.b).

All PHA-stimulated lymphocyte cultures in this report were incubated for a total period of 72 hr and radioactive thymidine was added for the final 24 hr of incubation.

Thymidine Concentration and Specific Activity

Measurement of DNA synthesis by the incorporation of radioactively labelled thymidine is the most widely used means of quantitating lymphocyte responses to stimulation. The two isotopes commonly used are tritium [^{3}H] and Carbon 14 [^{14}C] . Although [^{14}C] -thymidine is approximately 50 times more expensive than [^{3}H] -thymidine, only about one tenth of the amount of radioactivity is needed because the [^{14}C] counting efficiency is greater and quenching is less of a problem (Moorhead and McFarland, 1966; Ling and Kay, 1975). For this reason [^{14}C] -thymidine was selected as the isotope for this study.

A number of attempts have been made to optimize the accuracy of lymphocyte DNA labelling methods (Schellekens and Eijsvoogel, 1968; Sample and Chretien, 1971; Janossy, Greaves, Doenhoff and Snajdr, 1973).

Janossy *et al.* (1973) found that a linear relationship between responding cell numbers and incorporated thymidine was promoted when the extracellular thymidine concentration was constant during the

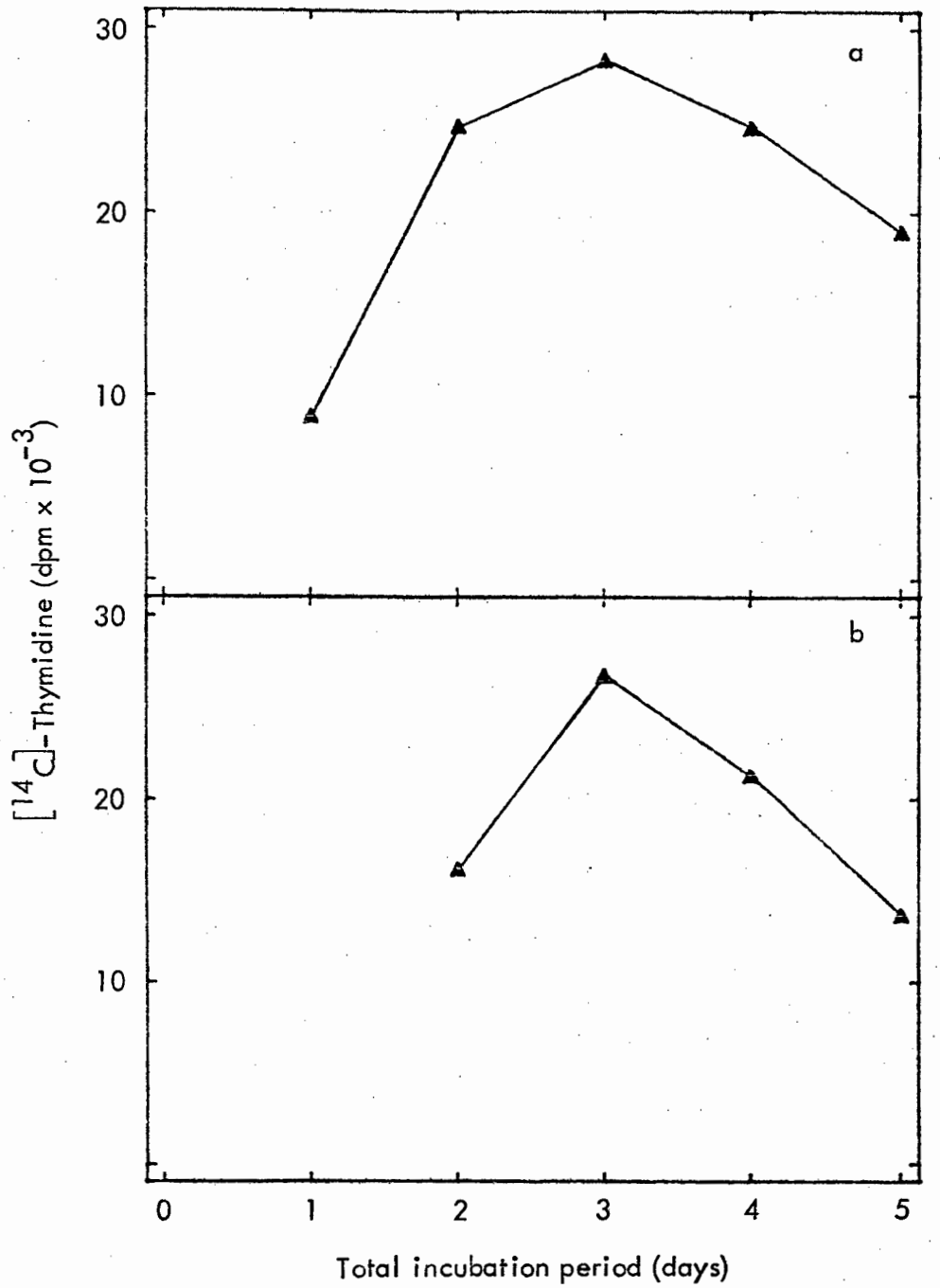


FIGURE 2.8 The effect of incubation time on the incorporation of radioactive thymidine by PHA-stimulated lymphocytes.

Triplicate cultures of PHA-stimulated lymphocytes in Nunc tubes (panel a) and microplate wells (panel b) were pulsed with $[^{14}C]$ -thymidine for the final 24 hrs of culture. Each point on the graphs represents the mean value of incorporated $[^{14}C]$ -thymidine in cultures harvested after the times indicated had elapsed.

Note that in both culture systems maximal incorporation of radioactivity was found after 3 days incubation.

FIGURE 2.9

FIGURE 2.9 The effect of the duration of radioactive pulse period on the incorporation of radioactivity by PHA-stimulated lymphocytes.

Triplicate cultures of PHA-stimulated lymphocytes in Nunc tubes (panel a) and microplate wells (panel b) were incubated for a total period of 3 days. Each point represents the mean values of radioactivity incorporated by cultures pulsed with $[^{14}C]$ -thymidine for the number of hours shown. The total incubation time for all cultures was 72 hours.

Note that in both culture systems maximal incorporation of radioactivity by PHA-stimulated lymphocytes was achieved when $[^{14}C]$ -thymidine had been present in cultures for 20 hours and that values obtained at 20 and 24 hours were similar.

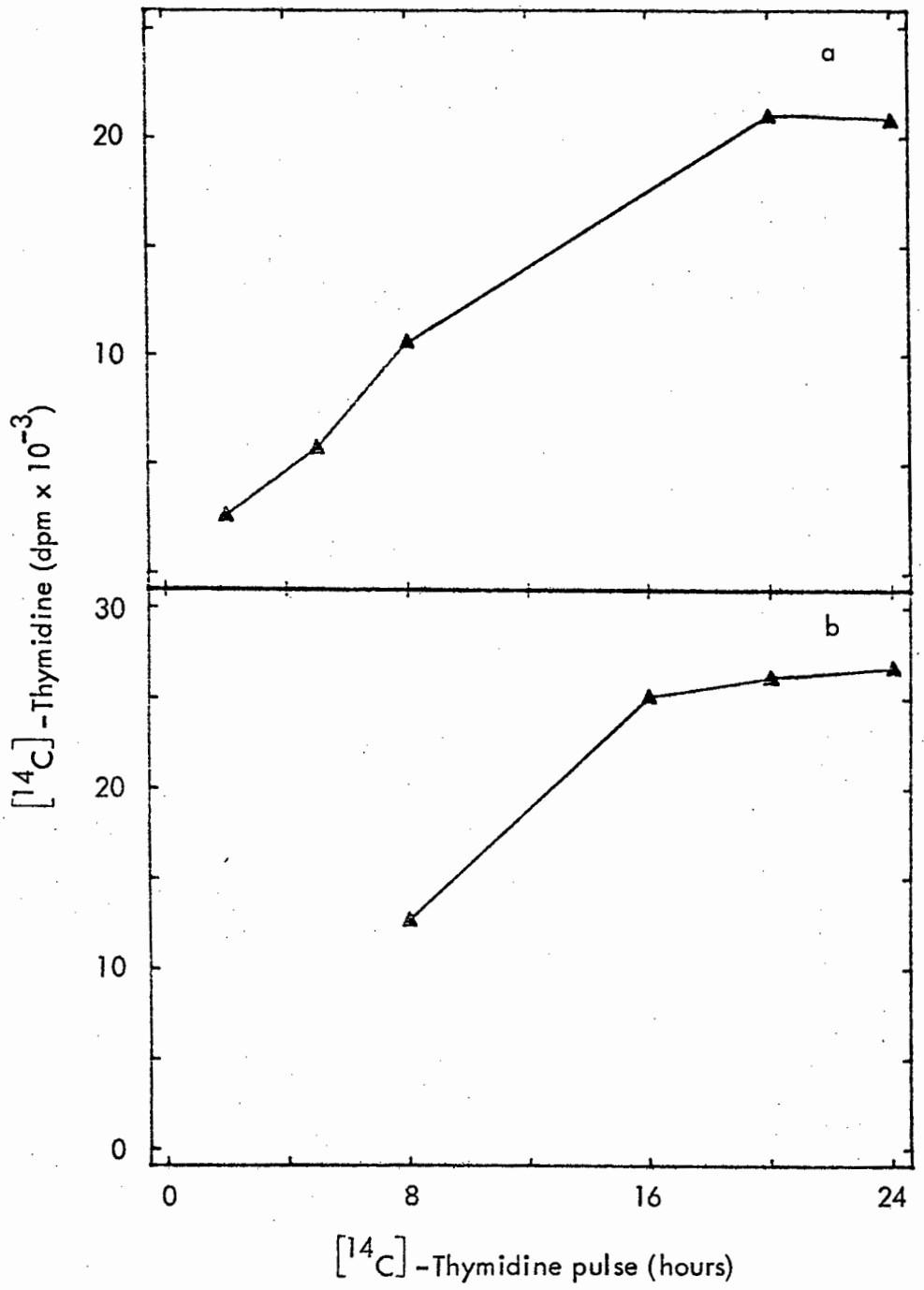


FIGURE 2.9 The effect of the duration of radioactive pulse period on the incorporation of radioactivity by PHA-stimulated lymphocytes.

labelling period. This was achieved by adding non-radioactive thymidine to maintain the concentration of thymidine between 5-20 µg/ml. Using a 1 ml culture volume with 5 x 10^5 lymphocytes, Sample and Chretien (1971) found that the concentration of thymidine necessary to saturate the system was 24,2 µg/ml. Du Bois *et al.* (1973) used a thymidine concentration of 2,9 µg/ml in their cultures.

In order to establish optimal labelling conditions for this culture system an experiment was conducted in which a constant amount of radioactivity (0,075 µCi of $[^{14}C]$ -thymidine) was added to all the culture tubes in the presence of varying amounts of non.radioactive thymidine. The amount of thymidine incorporated by 3 x 10^5 lymphocytes was calculated as follows:-

$$\mu g \text{ thymidine incorporated} = \frac{\text{dpm stimulated - dpm unstimulated}}{165\ 000} \times \mu g \text{ thymidine added,}$$

where 165 000 equals the number of disintegrations per minute present in 0,075 µCi of $[^{14}C]$.

The results of this experiment (Table 2.9) show that the system became saturated at a thymidine concentration of 20,29 µg/ml. This is in agreement with the results obtained by Sample and Chretien (1971) who used similar culture conditions. However, at this concentration the result of stimulated cultures was only 762 dpm. In order to achieve better counting statistics, considerably larger amounts of radioactive thymidine would have to be added. This is not an economically feasible proposition and I was accordingly compelled to use concentrations of thymidine that were rate-limiting. Since this imposed a stringent requirement for accurate dispensing of the radioactive compound, great care was taken with this step and commercially available $[^{14}C]$ -thymidine was used as supplied, without dilution with non-radioactive materials. The nucleoside was therefore added at a specific activity of approximately 60 mCi/mMole.

TABLE 2.9

THE INCORPORATION OF THYMIDINE BY 1 ml CULTURES CONTAINING 3×10^5 LYMPHOCYTES AND 0,075 µCi OF $[^{14}C]$ -THYMIDINE TO WHICH VARIABLE AMOUNTS OF NON-RADIOACTIVE THYMIDINE WERE ADDED.

Thymidine added (µg)	Specific activity (mCi/mMole)	dpm (stim.-unstim.)	Thymidine incorporated (µg)
0,29	62	27050	0,0428
1,54	11,8	7852	0,0660
2,79	6,5	4578	0,0697
5,29	3,43	2506	0,0723
10,29	1,76	1395	0,0783
20,29	0,89	762	0,0843
40,29	0,45	384	0,0844

Harvesting of Labelled Lymphocytes

Two basic methods have been used for separating radioactively labelled lymphocytes from unincorporated radioactivity in culture fluids: precipitation and washing of the lymphocytes by centrifugation and trichloroacetic acid (TCA) treatment, and separation by washing the cultures on glass fibre filter discs so that lymphocytes are retained and free radioactivity is washed away. Du Bois *et al.* (1973) compared these two methods and found that by simply washing lymphocyte cultures harvested on glass fibre discs with water they achieved the same results as they did when cells were precipitated and washed with TCA. Davies and Cocking (1966) and Davies and Hall (1969) have shown that liquid scintillation counting of samples collected on glass fibre discs is highly efficient and reproducible. The efficiency of counting using only small volumes of liquid scintillator (1-3 ml) is between 80-90% with dry $[^{14}C]$ samples, but self-absorption may be a problem with $[^{3}H]$ samples. They were unable to show any differences between Millipore and Whatmans glass fibre filter paper, and demonstrated that quench correction curves with glass fibre discs are perfectly satisfactory.

In order to see if their observations held true for this culture system, a large pool of radioactive lymphocytes was established. Six 1 ml aliquots were harvested by centrifugation, precipitation with cold 5% TCA and 100% methanol, and then transferred to counting vials with 5 ml scintillation fluid. A further six 1 ml aliquots were harvested on glass fibre filters as described in the methods section of this chapter and counted in a liquid scintillation spectrophotometer. The results shown in Table 2.10 indicated that there is a slightly higher reading with the precipitation method, possibly due to retention of unincorporated radioactivity in the pellet, but that there is little to choose

TABLE 2.10

MEASUREMENT OF $[^{14}C]$ -THYMIDINE INCORPORATION BY PHA-STIMULATED LYMPHOCYTE CULTURES USING EITHER TCA PRECIPITATION OR GLASS FIBRE FILTRATION

TCA precipitation	Glass fibre filtration
38877*	35944
40865	39239
39133	38835
40887	39684
41849	39526
41314	38888
Mean 40487	38686
Coeff. of variation 3,0	3,6

* Disintegrations per minute x 10^{-3}.

between the two systems.

This table also shows that the coefficient of variation was acceptably low and that there was no inherent variability in either harvesting technique.

To establish whether non-specific binding of $[^{14}C]$ -thymidine to the glass fibre filters occurred, 1 ml aliquots of culture fluid containing the standard amount of $[^{14}C]$ -thymidine but without lymphocytes were washed through filters. The counts obtained for 12 filter discs treated in this way were all less than 11 dpm.

When the multiple automatic sample harvester was first used for microplate harvesting, its efficiency was compared with the filter disc method of harvesting tube cultures.

A large pool of $[^{14}C]$ -thymidine-labelled lymphocytes was established and 200 µl aliquots were dispensed into Nunc tubes or into the wells of the microtitre plate. The culture fluid in the tubes was harvested using the millipore filtration apparatus, and the microplate cultures harvested using the automatic harvester with the filter paper positioned with either the smooth or the rough side up, and with the discs either punched out by the rings or cut out as squares of filter paper containing the ringed suction position. The results in Table 2.11 show that there was no difference between these harvesting techniques and that the automatic harvesting apparatus gave exceptionally good reproducibility.

Liquid Scintillation Counting of Samples and Correction for Quenching to Disintegrations per Minute

Liquid scintillation counting of $[^{14}C]$ samples on glass fibre discs is simple, rapid and highly efficient and reproducible. The use of standard quenched samples and the construction of quench curves using the AES ratio of the standards for correction of samples on glass fibre

TABLE 2.11

COMPARISON OF VARIOUS HARVESTING METHODS USING GLASS FIBRE FILTER PAPERS FOR RADIOACTIVELY LABELLED LYMPHOCYTE CULTURES

Harvester	Smooth or rough side upwards	Cut out or punched out	dpm [^{14}C] -Thymidine (mean of 12 replicates)	Coefficient of variation
Millipore filtration apparatus	-	-	9878	2,4
MASH*	smooth	cut	9676	1,4
MASH	smooth	punched	9778	1,5
MASH	rough	cut	9657	1,0
MASH	rough	punched	9686	1,2

* MASH = Multiple Automatic Sample Harvester

discs to disintegrations per minute has been reported to be highly accurate (Davies and Cocking, 1966; Davies and Hall, 1969).

Using the equipment available (Packard Tricarb Liquid Scintillation Spectrophotometer, model 3380, and Packard standard quenched samples containing 100 000 dpm $[^{14}C]$) quench curves were constructed for each run of samples counted. A typical quench curve is shown in Fig. 2.10.

The AES ratio of the test samples can be fitted to this curve, the percentage efficiency of counting calculated and the counts per minute (cpm) corrected to disintegrations per minute (dpm). Raw counting data were recorded on punched tape and entered into a Hewlett Packard desk top calculator which was programmed to perform the necessary calculations. The percentage efficiency of counting of six samples harvested on glass fibre filters as calculated by this method is shown in Table 2.12.

Using the same samples the percentage efficiency was also calculated by adding an extra amount of $[1\text{-}^{14}C]$ -Hexadecane as an internal standard. 100,3 µl of this solution contained 18622 disintegrations per minute. These results are also shown in Table 2.12. The mean efficiency of the counting of 6 samples as calculated by the quench curve was 88,3% and the mean efficiency of the same samples as calculated by adding the $[1\text{-}^{14}C]$ -Hexadecane standard was 87,9%. These figures are in agreement with those of Davies and Hall (1969) who found the efficiency of counting $[^{14}C]$samples on glass fibre discs to be between 80-90%. In this study all cpm recorded were corrected to dpm using standard quenched $[^{14}C]$ samples and a quench curve.

Reproducibility

The experiments reported above were undertaken so that the lymphocyte transformation test could be standardised as far as possible. It has been possible to fully standardise and control various aspects of

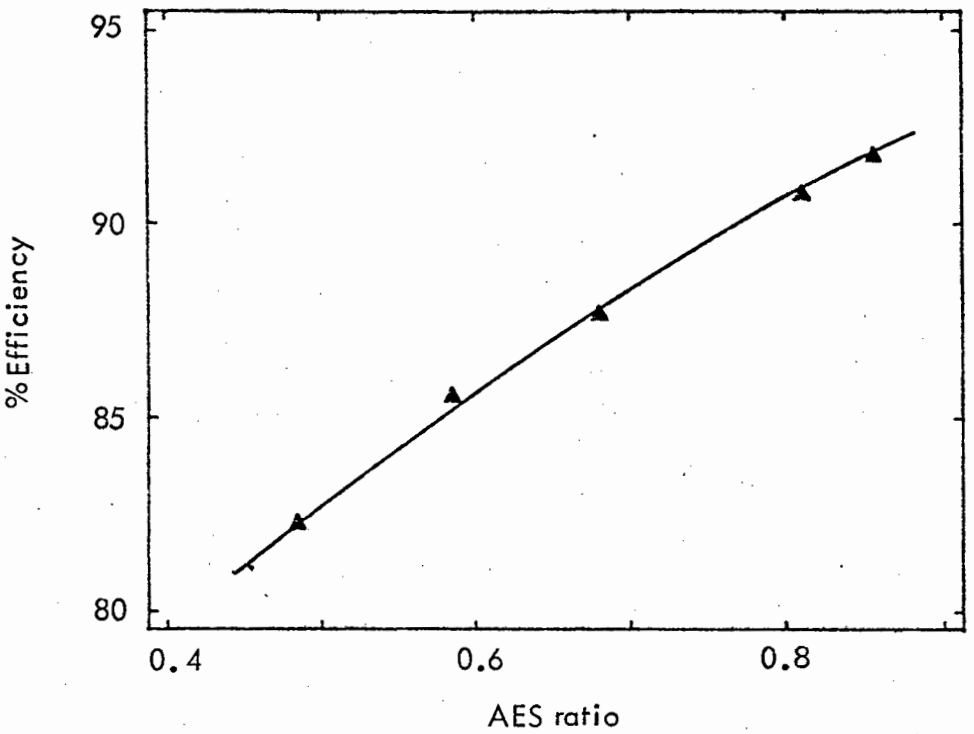


FIGURE 2.10 $[^{14}C]$ -quench curve

This graph shows the relationship between the Automatic External Standard (AES) ratio and the percentage efficiency of the quenched standard counts. A second-degree polynomial curve fits the plotted points.

TABLE 2.12

COMPARISON OF % EFFICIENCY OF COUNTING [^{14}C] SAMPLES ON GLASS FIBRE FILTERS USING THE AES RATIO AND A QUENCH CURVE, AND DIRECT MEASUREMENT WITH [1-^{14}C] -HEXADECANE.

Sample no.	1	2	3	4	5	6	Mean
AES ratio	0,6951	0,6902	0,6926	0,7021	0,7117	0,7020	
cpm	5463	5344	5400	6936	6952	6927	
% efficiency from quench curve	88,2	88,1	88,1	88,3	88,5	88,3	88,3
cpm after adding 18622 dpm of [1-^{14}C] -Hexadecane	21891	21539	22003	23209	23352	23302	
% efficiency calculated from addition of [1-^{14}C]-Hexadecane	88,2	86,9	89,2	87,4	88,0	87,9	87,9

this assay, namely:- the culture tubes, medium, buffers, mitogens, labelling compounds, incubation conditions, the harvesting and counting of radioactivity and the calculation of results. Room for error in the technique exists in the following areas:-

a) The separation and isolation of lymphocytes was not always completely satisfactory in that the possibilities existed that lymphocyte preparations might have been selectively enriched with one lymphocyte subpopulation and that a varying degree of contamination with red cells, polymorphonuclear leukocytes and monocytes was observed. Measures taken to minimise the adverse effects of these variables included the rejection of samples in which the lymphocyte yields were less than 50%, in which the viability was less than 95%, and in which contamination with polymorphonuclear cells was greater than 5%.

b) The accurate counting of lymphocyte numbers is essential. The use of defibrinated blood, an electronic cell counter and accurate dispensing apparatus ensured accuracy in this respect.

c) The serum source may vary in its supportive capacity. Since a universally standardised serum source is not available, a large pool of heat-inactivated serum from blood group AB Rh +ve donors was tested for cytotoxicity and ability to support lymphocyte transformation and stored at $-80^{o}C$.

d) "Control" lymphocytes from normal individuals show an inherent variability in their response to mitogens and other stimuli which cannot be standardised (Richter and Naspitz, 1967; Simons, Fowler and Fitzgerald, 1969; McIntyre and Cole, 1969; Ling and Kay, 1975). This makes it impossible to draw valid conclusions from differences in day-to-day results and difficult to define normal lymphocyte

transformation values. In each experiment in which a test subject was investigated a "normal" subject's lymphocytes were set up as a control. Although this solution was not ideal, it did provide a reference control value for each experiment.

Controls within each experiment were established by setting up all determinations in triplicate tubes. For each triplicate mean values and the coefficient of variation was obtained.

Using this means of checking variation in each test, Schellekens and Eijsvoogel (1968) found that 86% of their triplicate cultures of PHA-stimulated lymphocytes had a coefficient of variation of less than 15%. Penhale *et al.* (1974) reported that 93% of their triplicates had a coefficient of variation of less than 15% and Du Bois *et al.* (1973) had a mean coefficient of variation of 4,6% in PHA-stimulated cultures. The coefficient of variation obtained in the studies on the kwashiorkor patients and controls reported here were less than 15% in 81,5% of PHA-stimulated cultures in tubes with a mean of 10,2% in 135 triplicates. Although this is not as good as the reported figures for controls, these studies involved patients in whom PHA stimulation gave poor responses and hence low counts of $[^{14}C]$ -thymidine incorporation. The coefficient of variation depends on the calculation of the mean divided by the standard deviation. Therefore, if the standard deviation remains the same but the mean rises, the coefficient of variation will become smaller.

STANDARDISED METHODS FOR MEASURING LYMPHOCYTE TRANSFORMATION IN VITRO

This section describes the methods that were used for measuring lymphocyte transformation *in vitro*, based on the experiments reported in the preceding section.

Reagents:

Tissue Culture Medium (Eagles MEM)

Eagles Minimum Essential Medium for Suspension Cultures (Eagles MEM) buffered with 0,025 M Tris and with antibiotics was used throughout in the preparation of lymphocyte suspensions, and the establishment of cultures for lymphocyte stimulation. One packet of powdered Minimum Essential Medium (Eagle) for Suspension Cultures with Spinner Salts and L-glutamine (Gibco, U.S.A., cat. no. F-14) was dissolved in approximately 900 ml of triple distilled water. Three grams of Tris (Tris (hydroxymethyl)-amino methan, Merck, cat. no. 8382) were added and the pH adjusted to 7,3 with 0,1 N hydrochloric acid or sodium hydroxide. The volume was brought up to 1 000 ml in a volumetric flask and filtered by negative pressure through a 0,45 µ filter (Millipore, HAWP 0 47 00). The medium was sterilely decanted into 100 ml screw-cap bottles and stored at $4^{o}C$ for up to 4 weeks. Antibiotic solutions were added to the Eagles MEM just prior to use to give a final concentration of 100 u of penicillin and 100 µg of streptomycin per millilitre.

Ficoll Isopaque Lymphocyte Separation Mixture

The formula for the preparation of Ficoll-Isopaque was derived from the method as described by Böyum (1974).

56,47 grams of Ficoll 400 (Pharmacia) was dissolved in approximately 750 ml of triple distilled water. 128,62 ml of 75% Isopaque (sodium metrizoate solution, Nyegaard and Co, Oslo) was added and the volume made up to 1 000 ml in a volumetric flask.

Using a Zeiss refractometer model 47156, the density of the Ficoll-Isopaque separation mixture was measured as the percentage sucrose content. A standard curve was prepared for this determination by plotting the density of Ficoll Isopaque as determined by gravimetric analysis with density bottles, against the percentage sucrose reading on the refractometer (Fig. 2.1). A specific gravity of 1,077 at room temperature ($20^{o}C$) was equivalent to a sucrose content of 15,3%.

The adjustment of specific gravity to coincide with this reading was achieved by adding distilled water or 75% Isopaque as necessary.

The corrected solution was filtered through a 0,45 µ millipore filter by negative pressure and decanted into 100 ml screw-cap bottles. The solution was stored at room temperature, protected from light, and used within 2 weeks of preparation.

Serum for Cell Cultures

To obtain a standard source of serum (AB serum), one unit (approximately 250 ml) of serum was obtained from each of four healthy young male donors with blood group AB Rh +ve. The serum was heat-inactivated at $56^{o}C$ for 30 minutes, pooled and passed through a 0,45 µ millipore filter by positive pressure. The pooled, heat-inactivated, sterile serum was dispensed in 3 ml aliquots into sterile polypropylene plastic tubes (Falcon, cat. no. 2063) and stored at $-80^{o}C$ until used. A large

batch such as this lasted between 6-12 months without apparent loss of supportive activity. Only freshly thawed aliquots were used for lymphocyte culture and the residue was discarded. New batches were compared with the existing supply to ensure that there were no differences in their ability to support lymphocyte transformation.

Autologous serum was prepared by allowing venous blood to clot in a glass test tube for 1-2 hours at room temperature. The tubes were then centrifuged at 1 000 g for 10 min, the serum was removed, heat-inactivated at 56°C for 30 min, and filtered through a 0,45 µ millipore filter into sterile plastic test tubes (Falcon, cat. no. 2063). Serum that was not used immediately was stored at -80°C.

Phytohaemagglutinin (PHA)

Purified phytohaemagglutinin (Wellcome, cat. no. HA 16) was used in all experiments.

The 2 mg vial of dried phytohaemagglutinin was reconstituted with 2 ml of sterile saline and stored at -20°C for up to 3 months. A 1 in 50 dilution in medium was prepared just prior to use for the standard assay.

Concanavalin A (Con A)

Concanavalin A (Calbiochem, cat. no. 234567) was supplied as a dried preparation. Ten ml of sterile water was added to the vial to give a concentration of 25 mg per millilitre. The reconstituted solution was stored in small aliquots at -80°C and appropriate dilutions were made in medium as needed.

Pokeweed Mitogen (PWM)

Ten ml of distilled water was used to reconstitute one vial of Pokeweed mitogen (Gibco; Barker and Fames extraction, cat. no. 536), to achieve a concentration of approximately 5 mg per millilitre. The

reconstituted solution was stored at -80°C in small aliquots and appropriate dilutions were made in medium when needed.

[^{14}C] -Thymidine

[2-^{14}C] -thymidine was obtained from the Radiochemical Centre, Amersham (cat. no. CFA 219) with a specific activity of 60 mCi per mMole. It was supplied as an aqueous solution containing 50 µCi per millilitre and stored at 4°C. Appropriate dilutions were made in medium prior to use.

Mitomycin C

Mitomycin C for the treatment of lymphocytes in the mixed lymphocyte reaction was obtained from A. Christiaens N.V., Brussels. Two mg of crystalline mitomycin C was reconstituted in 40 ml of medium to give a concentration of 0,05 mg per millilitre. This was stored at 4°C and remained stable for at least 4 weeks.

Culture Tubes and Plates

The separation and washing of lymphocytes was done in 16 x 125 mm screw-cap plastic tissue culture tubes (Falcon, cat. no. 3033).

For lymphocyte cultures in tubes NUNC 11 x 70 mm round-bottomed polystyrene tubes with polyethylene stoppers were used (A/G NUNC, Denmark, cat. no. 1090). These were supplied dust-free and were sterilised by exposure to ultraviolet light for 1 hour before use.

Lymphocyte cultures in microtitre plates were set up in sterile round-bottomed microtitre plates (Cooke, cat. no. M 220-24 AR) and covered with sterile lids (cat. no. 220-42 29 AR). During culture the plates were wrapped in thin plastic sandwich wrap which had been exposed to U.V. light (Jiffy wrap).

Dispensing Apparatus

For dispensing volumes of 0,3 to 1,0 ml a 1,0 ml Biopette (Schwarz-Mann, cat. no. 00 10-29) with gas-sterilised disposable polypropylene tips (cat. no. 00 10-30) was used.

For volumes of 0,025 to 0,2 ml a 0,2 ml Biopette (cat. no. 00 10-19) with gas-sterilised disposable tips (cat. no. 00 10-20) and an adaptor kit for 0,025, 0,050, 0,075 and 0,15 ml volumes were used.

For the addition of radioactive thymidine in the microplate cultures a 0,5 ml Hamilton syringe with a Teflon-tipped plunger, type 1750 SN, fitted to a Hamilton repeating dispenser, type PB 600-1, to dispense aliquots of 10 µl was used.

Defibrinated Blood

Aseptically obtained venous blood was collected into sterile glass bottles containing glass beads 8 mm in diameter and shaken either by hand or with a mechanical shaker for 10 min. A 20 ml screw-cap McCartney bottle containing 6 glass beads was suitable for defibrinating between 5-15 ml of blood.

A total white cell count was done on the defibrinated blood either with a haemocytometer or using the Coulter electronic counter and a smear prepared and stained with Leishman's stain for differential counting of the white cells. The total number of lymphocytes in the blood sample was calculated from these values.

Separation of Lymphocytes from Defibrinated Blood

The method used was originally described by Böyum (1968b) and later adapted and revised by himself and others (Thorsby and Bratlie, 1970; Böyum, 1974). The procedures were all performed with care to preserve sterility and separation and centrifugation steps were conducted at room temperature.

Ten millilitre of a mixture of equal volumes of defibrinated blood and Eagles MEM was carefully layered above 5 ml of Ficoll-Isopaque mixture in a 16 x 125 mm plastic tube using a pasteur pipette to prevent mixing at the interface. The tube was centrifuged for 15 min at a gravitational force of 800XG measured at the interface.

After centrifugation the buffy layer of cells at the interface was removed with a pasteur pipette and placed in a second 15 ml tube and suspended in 10-15 ml of Eagles MEM by dispersing the cells with a pasteur pipette. The cells were spun down to a pellet by centrifuging for 10 min at 400XG and the medium was discarded. This washing procedure was repeated once more and the cells were then resuspended in exactly one millilitre of Eagles MEM. A total white cell count, a differential count, and staining of a specimen with Trypan blue (see Appendix) were performed. The yield of lymphocytes, their purity, and their viability were established and the cells diluted with Eagles MEM to an appropriate concentration.

In the experiments described in this study lymphocyte separation and subsequent cultures were always performed on freshly obtained defibrinated blood.

Mitogen-Stimulated Cultures in Test Tubes

Lymphocytes were adjusted to a concentration of $3,8 \times 10^5$/ml in Eagles MEM. 0,8 ml of this suspension was dispensed into Nunc 11 x 70 mm test tubes. 0,2 ml of serum was added to achieve a concentration of 3×10^5 lymphocytes in 1 ml of culture fluid consisting of 20% serum in Eagles MEM. 0,05 ml of phytohaemagglutinin diluted in Eagles MEM to contain 1 µg per 0,05 ml was added to appropriate tubes. To those cultures not receiving PHA, 0,05 ml of plain Eagles MEM was added. The tubes were sealed, inverted to mix, and placed in a 37^oC incubator.

In each experiment patient's lymphocytes or control lymphocytes were cultured in the presence of AB or patient's serum, with or without PHA. Triplicate cultures for each lymphocyte : serum : mitogen combination were established.

After 48 hours of incubation, 0,05 ml of $[^{14}C]$ -thymidine was added to each tube.

The aqueous solution of $[^{14}C]$ -thymidine was diluted 33,3 times in Eagles MEM to achieve a concentration of 0,075 µCi per 0,05 ml.

The contents of the tubes were mixed and incubated for a further 24 hours at $37^{o}C$.

The procedure for setting up cultures with Concanavalin A or Pokeweed mitogen was identical to that used with PHA as the mitogen. Dose response curves of both these mitogens showed that optimal stimulation was achieved at a concentration of 25 µg per millilitre (Fig. 4.1). Maximal stimulation of lymphocyte cultures with Pokeweed mitogen was only achieved after a total of 5 days incubation and cultures with this mitogen were incubated for a total period of 6 days.

Mixed Lymphocyte Cultures in Test Tubes

In the mixed lymphocyte reaction the antigenic differences between two different lymphocyte suspensions stimulate the lymphocytes to transform, and this can be measured as the incorporation of radioactive precursor thymidine into the DNA of the transformed cells (Bain, Vas and Lowenstein, 1964). One-way mixed lymphocyte cultures require metabolic inactivation of the lymphocytes from one donor by incubation of the cells with mitomycin C (Bach and Voynow, 1966).

Metabolic inactivation of half the lymphocytes from both donors was achieved in the following way. Half of the defibrinated blood

was mixed with an equal volume of medium containing mitomycin C to give a final concentration of 0,025 mg per millilitre, and incubated in a 37°C waterbath for 30 minutes. The remaining blood was mixed with medium containing no mitomycin C and incubated in the same way. The separation and counting of lymphocytes then proceeded as described earlier and both samples were brought to a concentration of $3,8 \times 10^5$ lymphocytes per millilitre.

Mixed lymphocyte cultures were established according to the method of Du Bois *et al.* (1973). $1,5 \times 10^5$ responder (0,4 ml) and $1,5 \times 10^5$ mitomycin-treated stimulating cells (0,4 ml) in various combinations and 20% serum (0,2 ml) were incubated at 37°C for a total of 6 days. Twenty four hours before harvesting 0,075 µCi of $[^{14}C]$ -thymidine was added to each culture.

The following combinations of cell suspensions were usually set up from 2 donors. Mitomycin-treated cells are designated as such with a subscript m.

Donor A + Donor B_m Donor A_m + Donor B	one-way reactions
Donor A + Donor B	two-way reaction
Donor A_m + Donor B_m	negative control.

Mitogen-Stimulated Cultures in Microtitre Plates

Lymphocyte suspensions were adjusted to a concentration of $1,33 \times 10^6$ cells per millilitre in Eagles MEM, and 150 µl aliquots were dispensed into the wells of a microtitre plate.
25 µl of serum, and 25 µl of medium or medium containing PHA were added. Each well therefore contained 2×10^5 lymphocytes, 12,5% serum and 0,5 µg of PHA in a total volume of 200 µl. The contents of the wells were

mixed by tapping on the side of the plate, and the plate was covered with a plastic lid and wrapped in Jiffy wrap to minimise losses by evaporation. The plates were incubated in a 37°C incubator in a humid atmosphere. Incubation was interrupted at 48 hours for the addition to each well of 10 µl of a dilution of [^{14}C] -thymidine in Eagles MEM containing 0,075 µCi of radioactivity. The plates were incubated for a further 24 hours at 37°C. Optimal amounts of Con A in this system were between 5-10 µg per well and, in the case of PWM, between 10-20 µg per well.

Mixed Lymphocyte Cultures in Microtitre Plates

Seventy five microlitre volumes of allogeneic cell suspensions at a concentration of 1,33 x 10^6 per millilitre in Eagles MEM were added to each well followed by 25 µl of serum and 25 µl of Eagles MEM. There were thus 1 x 10^5 lymphocytes of each type present per culture. Incubation at 37°C was continued for a total period of 6 days with the addition of 0,075 µCi [^{14}C] -thymidine 24 hours before harvesting. Mitomycin treatment and cell and serum combinations were done in the same way as described for the mixed lymphocyte reaction in tube cultures.

Harvesting of Lymphocyte Cultures in Test Tubes

After incubation the contents of the tubes were vigorously resuspended on a vortex mixer and poured into a Millipore filtration unit (cat. no. xx10.002500) fitted with a 25 mm diameter glassfibre pre-filter (Millipore cat. no. A.P. 200 2500). Six of these filter units were mounted on a manifold and suction was applied by means of a water tap suction apparatus to draw the excess fluid through and retain the cells on the filter. Three millilitre of normal saline solution was added to each tube, which was then agitated on a vortex mixer and decanted onto

the filter paper. This procedure was completed twice to ensure that all the cells were removed from the tubes. Excess radioactive thymidine which had not been incorporated into the DNA of the lymphocytes was removed by running 20 ml of deionised water through the filters. The damp filters were then transferred to glass scintillation vials (Packard, cat. no. 6 000 134). The open vials were placed in a drying oven set at 60^{o}C for 30 min to dry out the filters. Five millilitre of liquid scintillation fluid (Instagel, Packard, cat. no. 600 3063) was added, the vials were capped, wiped clean and the radioactivity counted using a liquid scintillation spectrophotometer.

Harvesting of Lymphocyte Cultures in Microtitre Plates

A multiple automatic sample harvester for harvesting the microtitre cultures was designed and constructed in the laboratory workshop. The cell suspensions from a row of 12 wells in the microtitre plate were sucked onto glass fibre filters and washed with water in a manner similar to that used to harvest tube cultures. A photograph of this apparatus is shown in Fig. 2.11. Water-tap vacuum could be applied, through a controlling valve, to a manifold containing 12 perforated base plates. A sheet of glass fibre filter paper (Skatron A.S., Norway) was laid on top of this and the upper half of the manifold clamped down on the paper. Each perforated base plate coincided with an "O" ring-sealed cavity in the upper block attached to a length of thin polystyrene tubing. The 12 lengths of tubing were connected to a manifold with 12 stainless steel cannulae which could be lowered into the wells of the microtitre plate. The contents of the individual wells could then be sucked up and filtered through the sealed filter paper held in the clamping manifold, which retained the cells and allowed the excess fluid to be aspirated. Washing of the individual wells was accomplished by allowing water to

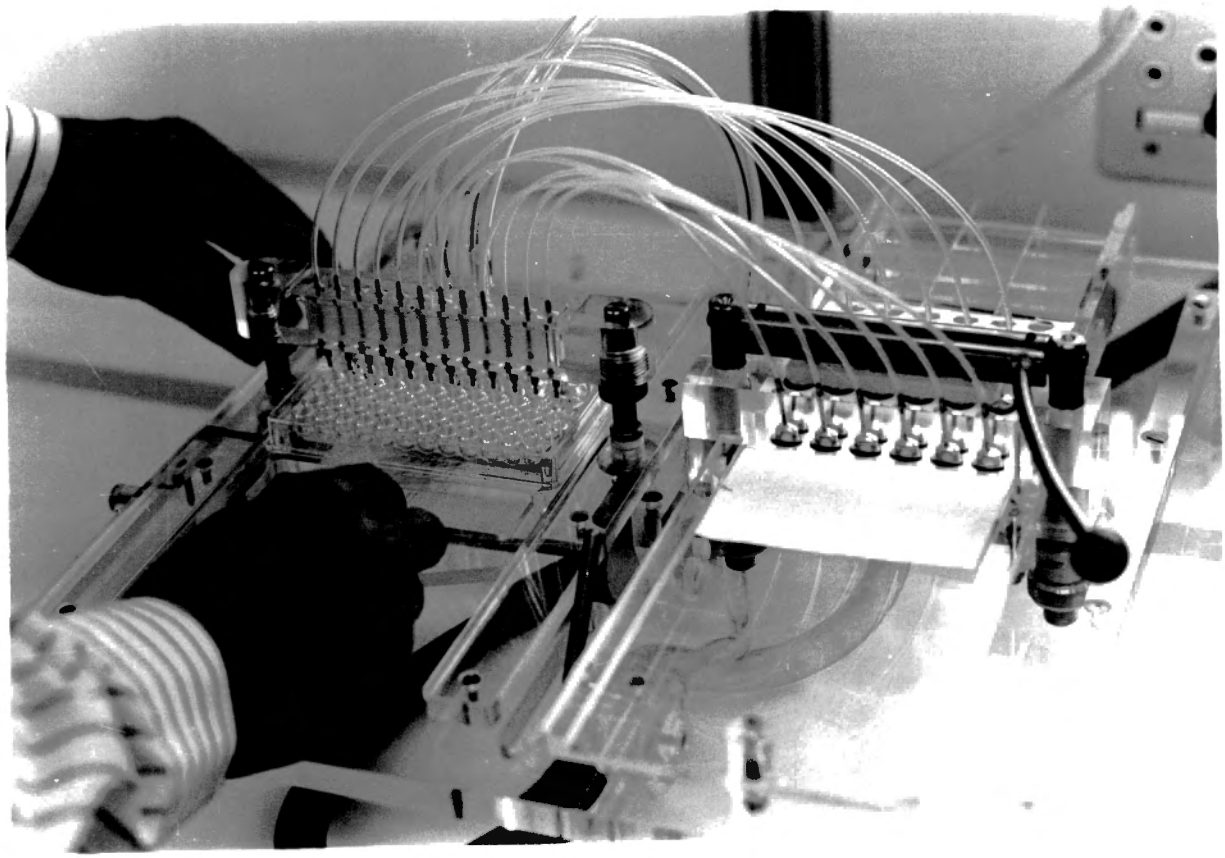


FIGURE 2.11 The Multiple Automatic Sample Harvester

This photograph shows the multiple automatic sample harvester used for harvesting lymphocytes cultured in microtitre plate wells. On the left hand side the manifold containing the aspiration and washing cannulae is lowered into the wells of the microtitre plate. Aspirated material is passed through the filter sheet which is clamped in the right hand manifold. For each row of wells the microtitre plate and the filter sheet are advanced together. The flow of washing fluid and suction are controlled by a valve situated in the foreground beneath the base plate.

run into each well through the lumen of a second, shorter stainless steel cannula concentric with the suction cannula. Water entering the well through the outer lumen was thus sucked off through the inner lumen of the cannula and through the filters. The plate was agitated gently during this procedure to make sure that all the cells were freed from the base of the well. A combination control valve allowed for the independent control of suction alone, or washing with suction. After washing had been completed (30 secs) the wells were sucked dry, the filter holding manifold was released, and the sheet of filter paper advanced to repeat the process for the next row of culture wells. When all the wells had been harvested the sheet of filter paper was dried. The discs, formed by the fracture of the glass-fibres by the "O" ring sealing pressure, were easily pushed out into the glass scintillation counting vials. Two millilitre of liquid scintillation fluid (Instagel) was added and radioactive counting proceeded with.

Radioactivity Counting and the Calculation of Results

Samples were counted in a Packard Tricarb liquid scintillation spectrophotometer (model 3380). The samples were processed in the following sequence: one vial without radioactivity for background counting, $[^{14}C]$ -standards with various degrees of quenching and fixed radioactivity content (100 000 disintegrations per minute) and the test samples in triplicate. The samples were counted for 5 minutes on the $[^{14}C]$ -channel with the machine set for measuring the Automatic External Standard Ratio (AES). The results were printed out and punched onto paper tape.

The data were fed into a programmable desktop calculator (Hewlett Packard Series G800 Model 10 with Printer Alpha Rom and Mathematics Block accessories), by means of a tape reader (Hewlett Packard 9863A

Model 63). The calculator was programmed to subtract the background sample reading from all samples and to fit a second-degree polynormal curve to the relationship between the efficiency of the quenched standard counts and the AES ratios (Fig. 2.10). The counting efficiency for each test sample was obtained by interpolation of the AES ratio on the standard curve and test sample counts were thereby corrected to disintegrations per minute, and printed. After each set of triplicate samples, the arithmetic mean and the coefficient of variation for that triplicate was calculated and printed. The results were thus expressed as the arithmetic mean of the disintegrations per minute (dpm) of radioactivity of triplicate cultures and the coefficient of variation served as a control on the variation occurring within the triplicate cultures.

CHAPTER 3

IMMUNE FUNCTION STUDIES IN CHILDREN WITH KWASHIORKOR

INTRODUCTION

As discussed in the first chapter, there is considerable evidence to indicate that immune defence mechanisms may be altered by malnutrition. This study was confined to an investigation of immune function in children suffering from kwashiorkor or marasmic kwashiorkor. These disorders are well-defined and well-recognised clinical entities that are associated with a high mortality and morbidity.

Although the major part of this thesis is concerned with an investigation of the *in vitro* function of peripheral blood lymphocytes in kwashiorkor, other aspects of immune function were studied in less detail.

Details of the patients and controls, and the methods used to study the subjects, as well as the management and treatment of the patients are described in this chapter.

The results obtained are discussed and compared with the published findings of other investigators.

SELECTION OF PATIENTS

The children studied were all classified as having kwashiorkor or marasmic kwashiorkor according to the recommendations of the Wellcome Working Party on Malnutrition (Lancet Editorial, 1970). This simple classification was accepted by the Joint F.A.O./W.H.O. Expert Committee on Malnutrition (1971) and is shown in Table 3.1.

In addition, children were scored according to the system proposed by McLaren, Pellet and Read (1967) in which points are given for the presence of oedema, dermatosis, hair change and hepatomegaly, and for the serum albumin levels (Table 3.2).

TABLE 3.1

CLASSIFICATION OF PROTEIN-CALORIE MALNUTRITION SUGGESTED BY THE WELLCOME WORKING PARTY (LANCET EDITORIAL, 1970)

Weight	Oedema	
(% of standard*)	Present	Absent
80-60	Kwashiorkor	Undernourished
<60	Marasmic-Kwashiorkor	Marasmus

* Standard = 50th Boston Percentile (Vaughan, 1975).

Using this system, McLaren *et al.* (1967) found that children with marasmus scored 0-3, those with marasmic kwashiorkor 4-8, and those with kwashiorkor 9-15 points.

Children were excluded from the study if they had other active disease, or a recent history of such disease, that might complicate interpretation of the results obtained. Tuberculosis, measles, septicaemia, shock, severe anaemia, severe diarrhoea with dehydration, evidence of cardiac decompensation or any other condition requiring urgent, specific treatment, were examples of disqualifiers in this category.

Children were also excluded or withdrawn if it was felt, at any stage, that the requirements of the study would compromise therapeutic need, convenient management of the clinical condition, or the welfare of the child.

Permission to conduct this study was obtained from the University of Cape Town's Faculty of Medicine Ethical Review Committee; from the Department of Paediatrics, and from the Medical Superintendent of Red Cross War Memorial Children's Hospital.

Informed written consent to undertake investigations on each child was obtained from the parent(s). The nature of the tests was fully explained and the possible risks, discomfort or inconvenience involved were made clear before the signing of consent forms (see Appendix). The parents were free to refuse consent initially and free to withdraw their children from the study at any stage without prejudicing their chance of optimal medical care.

A total of 36 Black or Coloured children with severe malnutrition (30 with kwashiorkor and 6 with marasmic kwashiorkor) were investigated. All the children were examined by me and a full dietary, social and medical history obtained from the parent(s). Thirty two of the children

TABLE 3.2

SCORING SYSTEM FOR PROTEIN-CALORIE MALNUTRITION AS PROPOSED BY McLAREN ET AL. (1967)

	Points
Oedema	3
Dermatosis	2
Oedema plus Dermatosis	6
Hair change	1
Hepatomegaly	1
Serum albumin (g/100 ml)	
1.00	7
1.00 - 1.49	6
1.50 - 1.99	5
2.00 - 2.49	4
2.50 - 2.99	3
3.00 - 3.49	2
3.50 - 3.99	1
4.00	0

studied were admitted to the metabolic ward of the Red Cross War Memorial Children's Hospital. The remainder were patients admitted to the general wards or seen as outpatients at the hospital.

Follow-up investigations at 14 days, 28 days and 42 days after nutritional therapy had been started were undertaken when this was possible.

The total number of investigations done is shown in Table 3.3. Serum protein electrophoresis, a full blood count, and the effect of the patient's serum on allogeneic lymphocyte responses to PHA were studied in all subjects. Because of the limited volume of blood that could be taken at one time, not all of the other investigations were completed on every patient. In some cases serum obtained on admission was stored at -80^{o}C for investigation of the effects of kwashiorkor serum on lymphocyte transformation as described in Chapter 4.

Details of the clinical findings and the results of the investigations in each patient are given in the Appendix.

TREATMENT OF PATIENTS

The principles of treatment followed for children with malnutrition were:- the supply of sufficient protein and calories to restart growth; the correction of commonly associated deficiencies such as fluid, glucose, potassium, vitamins, iron and folic acid; and the treatment of associated infections.

Refeeding of protein and calories was introduced gradually; the practice in this hospital is to use a Casilan Cream formula initially, as lactose intolerance is often a problem (Bowie, Barbezat and Hansen, 1967). The feeding routines employed were as follows:-

TABLE 3.3

INVESTIGATIONS DONE ON THE 36 PATIENTS WITH KWASHIORKOR AND MARASMIC KWASHIORKOR

	Number tested on			
	Admission	14 days	28 days	42 days
Serum protein electrophoresis	36	9	9	5
Serum immunoglobulins (IgG, IgM and IgA)	34	9	9	5
Serum IgE	28	-	-	-
Serum C3	32	9	9	5
PHA lymphocyte transformation	12	9	6	5
Serum effect on PHA transformation of allogeneic lymphocytes	36	-	-	-
Mixed lymphocyte reaction	8	6	6	-
T + B cell rosettes	7	-	-	-
Delayed hypersensitivity skin tests to PPD and Candida	15	-	7	-
DNCB sensitisation	13	-	6	-
Isoagglutinin titres	14	-	-	-
NBT test	15	-	-	-
Full blood count	36	-	-	-

Day 1 - Half-strength Darrow's Solution with 2,5% dextrose water

Day 2 - 5 - Casilan Cream diluted with an equal volume of half-strength Darrow's Solution with 2,5% dextrose water

Day 5 - 9 - Full strength Casilan Cream (70 cal/100 ml)

Day 9 - 13 - Full strength Casilan Cream plus Nutrine

Day 14 - Full cream milk plus toddler's diet

The 24-hour requirement for 100-120 ml/kg was given as 3-hourly oral feeds (or by nasogastric tube if necessary).

With recovery, usually in the 2nd or 3rd week, the need for frequent feeding had disappeared.

Correction of dehydration was done orally as far as possible. Intravenous infusions of half-strength Darrow's Solution in 2,5% dextrose water were given when essential, and carefully monitored to prevent over-transfusion. In practice most children maintained a satisfactory state of hydration on the feeding regime given above, but extra oral half-strength Darrow's Solution in 2,5% dextrose water was given to make up any extra losses or estimated deficits as they occurred.

All the children were given supplementary oral potassium chloride at a dosage of 3 meq/kg/day for the first 10 days of their admission and for longer if there was continuing diarrhoea. All the children also received Vidaylin multivitamin syrup 5 ml daily throughout their stay; supplementary iron (ferrous sulphate 6 mg/kg/day) and folic acid (5 mg/day) were commenced after the 14th day of treatment.

The practice in this unit is to give routine antibiotic treatment to all patients with kwashiorkor and marasmic kwashiorkor. For the first seven days the children in this study all received, by intramusular injection, Procaine Penicillin 25 000 u/kg/day and Kanamycin sulphate 10 mg/kg/day in two divided doses.

Infections occurring later in their hospital stay were treated on the basis of bacteriological isolates and clinical findings, with appropriate antibiotics. Parasitic infestation was treated when present with appropriate agents.

PAEDIATRIC CONTROLS

With the permission and cooperation of the Medical Officer of Health for Cape Town and the Sister-in-charge of the Heideveld Child Welfare Clinic, and with the informed consent of the children's mothers, various parameters of immune function were investigated in 10 age-matched Coloured children attending this clinic who were healthy and well-nourished at that time.

The investigations done on these children are shown in Table 3.4. Details of the clinical findings and results of the investigations in each control child are given in the Appendix.

METHODS

Serum Protein Measurement

Total Protein was measured by the Biuret method (Henry, Sobel and Berkman, 1957) and electrophoresis was done on cellulose acetate micro-electrophoretic strips. Quantitative analysis of the albumin and globulin fractions was done by densitometry.

Full Blood Counts

These were performed in the hospital haematology laboratory on a Coulter electronic cell counter and differential counts were made on Leishman stained smears.

TABLE 3.4

INVESTIGATIONS DONE ON 10 WELL-NOURISHED PAEDIATRIC CONTROL SUBJECTS

	No. tested
Serum protein electrophoresis	10
Serum immunoglobulins (IgG, IgM and IgA)	10
Serum IgE	8
Serum C3	10
Lymphocyte transformation response to PHA	10

Blood Grouping and Isohaemagglutinin Titres

These tests were also performed in the hospital haematology laboratory using standard blood grouping and serological techniques.

Nitroblue Tetrazolium Test

One millilitre of blood was collected in heparinised tubes (20u/ml). Nitroblue Tetrazolium Dye (Sigma, cat. no. N 6876) was dissolved in 0,15 M NaCl to a concentration of 0,2%. This was then mixed, just prior to testing, with an equal volume of phosphate-buffered saline, pH 7,2. *E. coli* lipopolysaccharide Endotoxin (Difco, cat. no. 3920) was dissolved to a concentration of 0,002% in 0,15 M phosphate-buffered saline, pH 7.2.

The test was performed as follows:-
4 drops of heparinised blood were added to 2 drops of the NBT solution in phosphate-buffered saline and either 2 drops of the buffer alone, or 2 drops of the buffer solution containing endotoxin, and mixed. The suspension was incubated in a waterbath at 37^{o}C for 15 min and then allowed to stand at room temperature for a further 15 min. Smears were made and stained with a May-Grunewald Giemsa stain. Two hundred polymorphonuclear cells were examined and the number containing black formazan deposits counted.

Serum Immunoglobulins

Serum immunoglobulin IgG, IgM and IgA were measured by the Single Radial Immunodiffusion technique of Mancini, Carbonara and Heremans (1965) on tripartigen immunodiffusion plates supplied by Behringwerke A.G. (cat. no. OTDS, OTDT and ODTU). The standard serum samples used for these determinations were calibrated against reference serum 67/97, obtained from the W.H.O. International Reference Centre for Immunoglobulins, Switzerland, and were stored in small aliquots at -80^{o}C until used.

IgE determinations were done using a double antibody radioactive radial immunodiffusion technique, as described by Orren and Dowdle (1975). The lower level of sensitivity of this method is 25 u/ml.

Serum C3 Determination

The concentration of C3 was measured by single radial immunodiffusion in C3 M-Partigen plates obtained from Behringwerke A.G. (cat. no. OTBC). The standard employed in this assay was one supplied by the manufacturers, Protein Standard Serum B (cat. no. OTFG 07).

Delayed Hypersensitivity Skin Tests

These were performed according to the recommendation of the W.H.O. Committee on Primary Immunodeficiency (Fudenberg *et al.*, 1971). 0,1 ml of the appropriate antigen was injected intradermally into the volar aspect of the forearm and the degree of induration read at 24 and 48 hours. At each time the diameter of induration was measured at right angles; the mean diameter of induration was calculated and the highest value obtained on any day taken as the response to the antigen.

The reagents used were:-

a) Purified Tuberculin (PPD) 5 T.U./0,1 ml, obtained ready for use from the Staten's Serum Institut, Copenhagen, Denmark.

b) Candida Antigen. *Candida albicans* 1/10; w/v in 50% glycerin was obtained from Hollister Stier Laboratories, Spokane, Washington. This was further diluted 1:10 in sterile buffered saline to give an antigen concentration of 1/100. The 1/100 dilution is stable for 3 months when kept at $4^{o}C$.

Dinitrochlorobenzene Sensitisation

Dinitrochlorobenzene (DNCB) sensitisation was performed according to the method of Catalona, Taylor, Rabson and Chretien (1972). DNCB (Analar grade B.D.H.) was dissolved in acetone to form a stock solution of 2 000 µg per 0,1 ml and from this a further dilution in acetone of 50 µg per 0,1 ml was made. Sensitisation was performed by the application of 0,1 ml of the 2 000 µg/0,1 ml solution over a 3 cm^2 area on the forearm. The acetone was evaporated by a stream of air and an occlusive dressing applied for 24 hours. The challenge dose of 50 µg per 0,1 ml was applied at a different site in the same fashion 14 days later and the site examined at 24 and 48 hrs for induration. Any signs of induration at 24 or 48 hrs were recorded as positive.

Peripheral Blood T and B Cell Identification

Peripheral blood lymphocytes were characterised as T or B cells according to their ability to form rosettes with uncoated sheep red blood cells (SRBC) or with antibody and complement-coated SRBC respectively according to the method of Jondal, Holm and Wigzell (1972) with minor modifications.

Lymphocytes were separated on a Ficoll-Isopaque gradient as described in Chapter 2 and brought to a concentration of 4 x 10^6/ml in Hank's Balanced Salt Solution (HBSS) (Gibco, U.S.A.). SRBC were collected each week from the same sheep and stored in Alsever's solution at 4°C. Before use the cells were washed twice with HBSS.

a) Test Procedure for E Rosettes (T Cells). 0,25 ml of the lymphocyte suspension was mixed with 0,25 ml of 0,5% washed SRBC (approximately 80 x 10^6 SRBC/ml) and incubated at 37°C for 5 min in small glass test tubes. The cell suspension was spun at 200g for 5 min and incubated at 4°C overnight. The supernatant was

removed and the cell pellet gently resuspended by shaking. One drop of the cell suspension was mounted on a glass slide under a cover slip and sealed with Vaseline. Two hundred lymphocytes were counted using phase contrast optics and all lymphocytes binding more than three SRBC were scored as rosettes.

b) Test Procedure for EAC Rosettes (B Cells). Five millilitres of a 5% suspension of SRBC in HBSS was added to 5 ml of HBSS containing 4 haemolytic units of Burroughs Wellcome rabbit anti-sheep haemolysin, and incubated for 30 min in a 37°C waterbath. The cells were washed three times in HBSS at 500g for 10 min and resuspended in 5 ml of HBSS. Five millilitres of a 1/20 dilution in HBSS of fresh human serum was added as a source of complement and the suspension incubated at 37°C for 30 min. The EAC cells were then washed three times in HBSS and brought to a concentration of 0,5% (approximately 80 x 10^6 SRBC/ml). This preparation was stable at 4°C for 1 week. 0,25 ml of the lymphocyte suspension and 0,25 ml of the EAC preparation were mixed in a small glass test tube and incubated at 37°C in a shaking waterbath for 30 min. A drop of the suspension was mounted under a cover slip, sealed with Vaseline and counted using phase contrast optics. Two hundred cells were counted and all lymphocytes binding more than 3 SRBC were scored as rosettes.

The patients' peripheral blood lymphocytes were always tested simultaneously with normal reference lymphocytes and each determination was done in duplicate.

Lymphocyte Transformation

PHA-stimulated lymphocyte transformation and mixed lymphocyte cultures were done in microplates as described in detail in Chapter 2.

It was not possible to establish lymphocyte transformation studies on the patients with kwashiorkor and the paediatric control subjects simultaneously. To overcome this, lymphocytes obtained from healthy laboratory personnel or from blood donors on the panel of the Western Province Blood Transfusion Service were incubated under identical conditions with either the patients' or control lymphocytes. These normal adult lymphocytes will be referred to as Reference lymphocytes. Likewise, Reference serum was the pooled blood group AB serum referred to, and prepared as described in Chapter 2.

RESULTS

Patients

Three of the 36 patients died within 4 days of admission (8% mortality). Autopsies were performed on 2 of these children and in both cases the gross changes seen in malnutrition as well as bronchopneumonia were present. In one case disseminated *Herpes simplex* infection was found. Two children were transferred to other hospitals and no further follow-up was available. Two children were not admitted as in-patients. Both showed clinical improvement on subsequent follow-up visits to the out-patients department.

The average duration of hospital stay for the remaining 29 children who were admitted to the metabolic ward was 4,3 weeks. All these children, except one who contracted measles and was transferred to the infectious diseases hospital, were well on discharge. Because of home circumstances, 8 were discharged to a convalescent hospital for a further period of nutritional therapy. A further 11 children from the local district were followed in the out-patients department for up to 18 months. Follow-up observations on these children showed satisfactory nutritional improvement in all except 3 cases who contracted respectively diarrhoea,

pneumonia and measles during this period. Follow-up of the 9 remaining children was not possible, either for geographical reasons or because of patient default. Summaries of the clinical, pathological and laboratory findings for each patient are included in the appendix.

Infections

Of the 32 patients admitted to the metabolic ward, 19 (59%) had one or more pathogens in their stools; 15 (47%) had either clinical or radiological signs of pulmonary infection; 11 (34%) had bacteriological evidence of infected skin lesions and 9 (28%) had bacteriological or clinical evidence of upper respiratory infections including *Otitis media.* No evidence of infection was found in only 3 children (10%), and infections at more than one site were present in 66% of the patients.

The numbers of children with positive stool isolates for the following pathogens were:- *Salmonella* species 6; *Giardia lamblia* 5; *Candida albicans* 4; *Trichuris trichuria* 4; *Ascaris lumbricoides* 3; *Trichomonas* 2; and *Shigella* species and enteropthogenic *E. coli* 1 each. Eighty two percent of the positive skin isolates were either β haemolytic streptococci or *Staphylococcus aureus.*

Anthropometric and Nutritional Data

Analysis of the age, percentage expected weight for age, and serum protein measurements on the 36 patients with kwashiorkor or marasmic kwashiorkor and on the 10 paediatric control subjects are shown in Table 3.5. Statistical analysis of differences between these two groups was performed using the Student's T test. The average age of the control subjects was 3 months younger than the patients', but this was not statistically significant. In both groups the ratio of females was greater. Amongst the patients this ratio was 1,57:1 and in the control

TABLE 3.5

ANTHROPOMETRIC AND SERUM PROTEIN VALUES OF THE KWASHIORKOR PATIENTS AND THE PAEDIATRIC CONTROL SUBJECTS

	No. studied	Age (months)	F:M ratio	% Expected wt. for age	g/100 ml Total Prot.	Serum Alb.	α_1 glob.	α_2 glob.	β glob.	γ glob.
Kwashiorkor	36		1,57:1							
Mean		23,9		67,4	4,27	1,65	0,27	0,66	0,55	1,14
(SEM)*		(1,83)		(1,58)	(0,14)	(0,07)	(0,01)	(0,03)	(0,02)	(0,06)
Paed. Control	10		1,5 :1							
Mean		20,4		103,0	6,67	3,76	0,24	0,80	0,85	1,00
(SEM)		(3,56)		(4,01)	(0,13)	(0,11)	(0,03)	(0,02)	(0,03)	(0,08)
T**		0,87		8,24	12,37	16,31	2,22	3,99	7,74	1,35
p value		N/S		<.001	<.001	<.001	<.05	<.001	<.001	<.2

* Standard Error of the Mean

** Student's T value

group 1,5:1. The patients showed significantly lower values for percentage expected weight for age, total serum protein concentration, serum albumin concentration, α-2 globulin and β globulin levels. In the case of α-1 globulin levels and γ globulin, the patients' mean levels were higher than those of the control subjects, but the differences were less significant.

In Table 3.6 the results of the percentage expected weight for age and serum protein measurements on admission and at various times after recovery are shown and compared with the values obtained in the paediatric control subjects. By 14 days there was a rapid rise in the total serum protein, serum albumin, α-2 globulin and β globulin levels. Further smaller increases in serum albumin were seen over the following 28 days. The total serum protein levels at 42 days were higher than in the paediatric controls due, almost entirely, to elevated γ globulin levels. Although the γ globulin levels on admission were higher than those in the control subjects, with nutritional recovery there was a further increase, suggesting an enhanced ability to synthesize new antibody. α-1 globulin levels fell to within normal levels by 28 days and the β globulin levels showed a rapid rise in 14 days to higher than the control values, with a fall to within normal levels by 42 days.

Immunoglobulin and C3 Determinations

The results of IgG, IgM, IgA, IgE and C3 levels are shown in Table 3.7. Significant differences were present between the values obtained in the kwashiorkor patients and the paediatric controls, in that children with kwashiorkor had raised immunoglobulin levels and low C3 levels. The most striking elevations were those in the IgM, IgA and IgE classes.

During the course of nutritional treatment and recovery, immunoglobulin levels and C3 levels were measured in some patients at 14, 28

TABLE 3.6

PERCENTAGE EXPECTED WEIGHT FOR AGE AND SERUM PROTEIN LEVELS OF CHILDREN WITH KWASHIORKOR ON ADMISSION AND AT VARIOUS PERIODS DURING RECOVERY

	Admission	14 days	28 days	42 days	Paediatric controls
No. tested	36	9	9	5	10
% Expect. wt. for age					
Mean	67,4	74,4	76,2	77,4	103
(SEM)*	(1,58)	(3,1)	(2,7)	(4,9)	(4,01)
Total serum prot.					
Mean	4,27	7,32	7,56	7,71	6,67
(SEM)	(0,14)	(0,15)	(0,14)	(0,22)	(0,13)
Albumin					
Mean	1,65	3,36	3,70	3,84	3,76
(SEM)	(0,07)	(0,10)	(0,06)	(0,12)	(0,11)
α_1 globulin					
Mean	0,27	0,28	0,23	0,25	0,24
(SEM)	(0,01)	(0,01)	(0,01)	(0,02)	(0,03)
α_2 globulin					
Mean	0,66	1,05	0,89	0,91	0,80
(SEM)	(0,03)	(0,08)	(0,05)	(0,11)	(0,02)
β globulin					
Mean	0,55	1,15	1,04	0,94	0,85
(SEM)	(0,02)	(0,01)	(0,05)	(0,05)	(0,03)
γ globulin					
Mean	1,14	1,47	1,69	1,78	1,00
(SEM)	(0,06)	(0,10)	(0,10)	(0,19)	(0,08)

* Standard Error of the Mean

TABLE 3.7

IMMUNOGLOBULIN AND C3 LEVELS IN PATIENTS WITH KWASHIORKOR AND PAEDIATRIC CONTROL SUBJECTS

	mg/100 ml				
	IgG	IgM	IgA	IgE (u/ml)	C3
Kwashiorkor					
No. tested	34	34	34	28	32
Mean	1243,7	184,7	154,2	604,6	77,4
(SEM)*	(87,6)	(13,3)	(11,9)	(146,9)	(7,2)
Paediatric control					
No. tested	10	10	10	7	10
Mean	937,1	122,4	81,0	69,6	154,9
(SEM)	(103,8)	(13,35)	(11,2)	(16,4)	(11,0)
T**	2,26	3,31	4,49	3,62	5,91
p value	<.05	<.005	<.001	<.001	<.001

* Standard Error of the Mean

** Student's T test

and 42 days. The results are shown in Table 3.8. With recovery IgG, IgM and IgA levels rose, especially in the first 2 weeks, with both IgM and IgA falling off at 4-6 weeks. At all times the immunoglobulin levels were higher than in control subjects. There was a rapid rise in C3 levels at 14 days to levels higher than those found in control subjects, followed by a fall to normal levels at 28 and 42 days. These changes in C3, a major β globulin protein, and in the individual immunoglobulin concentrations, were paralleled by the similar changes in β and γ globulin levels, as measured by electrophoresis (Table 3.5).

Isohaemagglutinin Serum Titres

All the 14 kwashiorkor patients tested had isohaemagglutinin antibodies against blood group substances A and B where applicable. The results are shown in Table 3.9. In some cases estimations were repeated at 4 weeks and a rise in isohaemagglutinin titres was noted.

Nitroblue Tetrazolium Test

The neutrophils of 15 patients tested were capable of reducing nitroblue tetrazolium. In all but 2 of these patients spontaneous reduction occurred without endotoxin stimulation. The results are shown in Table 3.9.

Peripheral Blood Counts

The mean values and ranges for peripheral blood haemoglobin, total white cell counts and total lymphocyte counts are shown in Table 3.10. Seven out of the 36 patients had total lymphocyte counts of less than 2 500 per mm^3. The only patient with a lymphocyte count of less than 1 000 per mm^3 died.

TABLE 3.8

IMMUNOGLOBULIN AND C3 LEVELS IN MALNOURISHED CHILDREN ON ADMISSION AND AT VARIOUS TIMES DURING NUTRITIONAL RECOVERY

	Admission	14 days	28 days	42 days	Paediatric controls
No. tested	34	9	9	5	10
IgG Mean	1243,7	1690	1839	1779	937
(SEM)*	(87,6)	(115)	(175)	(232)	(104)
IgM Mean	184,7	233	241	205	122
(SEM)	(13,3)	(26)	(29)	(27)	(13)
IgA Mean	154,2	195	169	114	81
(SEM)	(11,9)	(26)	(30)	(26)	(11)
C3 Mean	73,4	197	156	169	155
(SEM)	(7,2)	(7)	(14)	(10)	(11)

* Standard Error of the Mean

TABLE 3.9

BLOOD GROUP, ISOHAEMAGGLUTININ TITRES AND NITROBLUE TETRAZOLIUM REDUCTION IN PATIENTS WITH KWASHIORKOR

Case no.	Blood group	Isohaemagglutinin titre (reciprocals)		NBT Positive (%)	
		Anti A	Anti B	Without stimulation	With Endotoxin stimulation
1	AB+	-	-	21	N/D*
3	O+	512	256	70	N/D
4	O+	128	16	1	40
5	A+	-	128	24	N/D
8	N/D	N/D	N/D	36	N/D
10	O+	128	16	41	N/D
11	A+	-	16	44	N/D
12	O+	32	32	26	N/D
13	O+	64	64	10	28
14	A+	-	64	27	N/D
15	B+	4	-	14	N/D
16	O+	32	32	36	N/D
17	A+	-	64	75	N/D
18	O+	16	32	18	N/D
19	O+	512	32	16	N/D

* Not Done

TABLE 3.10

MEAN VALUES AND RANGE OF VALUES OBTAINED FOR PERIPHERAL BLOOD HAEMOGLOBIN CONCENTRATIONS, TOTAL WHITE CELL COUNTS AND TOTAL LYMPHOCYTE COUNTS IN 36 CHILDREN WITH KWASHIORKOR

	Haemoglobin g/100 ml	Total white cells per mm^3	Total lymphocytes per mm^3
Mean	8,86	11,570	4 244
Range	3,4-12,9	4,700-28,400	545-11 076

Delayed Hypersensitivity Skin Tests

a) PPD. Fifteen patients were tested on admission for delayed hypersensitivity responses to 5 TU of PPD. No patients gave a positive response. Seven were tested again at 28 days and no response was obtained. None of the children had clinical evidence of tuberculosis or had received BCG vaccinations.

b) Candida. None of the fifteen children tested with candida antigen gave an initial response with induration of greater than 10 mm diameter, but three had intermediate responses of between 5 and 10 mm induration. Three out of seven children with negative responses on admission showed an indurated lesion of greater than 10 mm at 28 days.

c) Dinitrochlorobenzene (DNCB). Thirteen children were sensitized with 2 000 µg of DNCB and tested for a delayed hypersensitivity response 10 days later with 50 µg of DNCB. Only one child gave a positive response. Of 6 initial non-responders, retested with a challenge dose at 28 days, only one child gave a positive response.

Lymphocyte Transformation Responses to PHA

a) On Admission

Results for two series of lymphocyte transformation experiments are given in Tables 3.11.a and 3.12.a and derived data for the corresponding 2-way analyses of variance are given in Tables 3.11.b and 3.12.b. These results are depicted graphically in Fig. 3.1. In the first series of 12 experiments (Table 3.11.a, Figs. 3.1.a and 3.1.b), PHA-induced radioactive thymidine incorporation into kwashiorkor and reference lymphocytes, each cultured in both patient and reference AB serum, were compared. The patient cells incorporated a mean of 7 844 dpm when incubated in autologous serum and

TABLE 3.11.a

PHA-INDUCED MITOGENIC RESPONSES OF PATIENT AND REFERENCE CELLS CULTURED IN THE COMBINATIONS SHOWN, IN THE PRESENCE OF AUTOLOGOUS OR POOLED AB SERUM. RESULTS GIVEN ARE THE MEAN dpm OF $[^{14}C]$ -THYMIDINE INCORPORATED BY TRIPLICATE CULTURES. VALUES FOR UNSTIMULATED CULTURES ARE SHOWN IN BRACKETS.

Cells:	Patient		Reference	
Serum:	Patient	AB	Patient	AB
Case 1	11453 (-)	48194 (135)	10233 (-)	36571 (88)
Case 2	9187 (614)	30082 (593)	5629 (182)	23052 (323)
Case 3	3865 (-)	23131 (200)	7631 (-)	24156 (44)
Case 4	13826 (836)	41870 (1033)	23722 (110)	38843 (197)
Case 5	4088 (139)	16227 (194)	15172 (155)	38509 (304)
Case 6	4259 (-)	11417 (-)	11835 (-)	22612 (-)
Case 7	17498 (273)	20411 (-)	11898 (-)	30206 (-)
Case 8	12479 (32)	53977 (-)	23019 (88)	52080 (-)
Case 9	3500 (281)	21686 (372)	4305 (111)	39238 (186)
Case 10	3728 (-)	14354 (304)	28756 (-)	29674 (186)
Case 11	6002 (-)	26762 (-)	6591 (-)	19305 (-)
Case 12	4243 (421)	19909 (613)	15927 (-)	29756 (75)
Averages	7844 (370)	27335 (430)	13727 (129)	32000 (175)

TABLE 3.11.b

TWO-WAY ANALYSIS OF VARIANCE OF DATA PRESENTED IN TABLE 3.11.a

Source of variance	D.F.	Sums of squares	M.S.S.	F	P
Sera	1	$4,29 \times 10^9$	$4,29 \times 10^9$	47,4	<0,001
Cells	1	$3,34 \times 10^8$	$3,34 \times 10^8$	3,7	N/S
Cells x sera	1	$4,45 \times 10^6$	$4,45 \times 10^6$	0,0	N/S
Remainder	44	$3,97 \times 10^9$	$9,03 \times 10^7$	-	-

TABLE 3.12.a

PHA-INDUCED MITOGENIC RESPONSES OF PAEDIATRIC CONTROL AND REFERENCE CELLS CULTURED IN THE COMBINATIONS SHOWN IN THE PRESENCE OF AUTOLOGOUS OR POOLED AB SERUM. VALUES AS FOR TABLE 3.11.a.

Cells:	Control		Reference	
Serum:	Control	AB	Control	AB
Control 1	50822 (-)	55180 (165)	22319 (-)	30326 (76)
Control 2	36129 (-)	46410 (214)	25136 (-)	33169 (180)
Control 3	28020 (1590)	25573 (1000)	24651 (1800)	32606 (1001)
Control 4	35290 (791)	32802 (411)	24428 (78)	25448 (1141)
Control 5	34595 (1229)	33593 (1027)	27444 (757)	27026 (592)
Control 6	30597 (-)	26726 (683)	32457 (-)	33144 (99)
Control 7	26213 (482)	25494 (518)	35639 (-)	37395 (203)
Control 8	28460 (583)	36887 (897)	24030 (506)	29730 (635)
Control 9	58804 (-)	58369 (266)	34283 (-)	38133 (162)
Control 10	23925 (83)	24190 (123)	23872 (-)	30206 (-)
Averages	35286 (793)	36522 (530)	27426 (785)	31718 (454)

TABLE 3.12.b

TWO-WAY ANALYSIS OF VARIANCE OF DATA PRESENTED IN TABLE 3.12.a

Source of variance	D.F.	Sums of squares	M.S.S.	F	P
Sera	1	$1,07 \times 10^8$	$1,07 \times 10^8$	1,4	N/S
Cells	1	$4,67 \times 10^8$	$4,67 \times 10^8$	6,0	0,05
Cells x sera	1	$1,06 \times 10^7$	$1,06 \times 10^7$	0,1	N/S
Remainder	36	$2,82 \times 10^9$	$7,83 \times 10^7$	-	-

a mean of 27 335 dpm in normal AB serum. Reference cells incorporated a mean of 13 727 dpm when cultured in the presence of patient serum and 32 000 dpm when cultured in AB serum. Inspection of the table reveals that the marked depressive effect of kwashiorkor serum on both patient and reference lymphocytes was evident, not only in the mean results but equally and consistently so in each individual experiment with the exception of case 7, where the patient's cells performed comparably in control and AB serum. As can be seen from the mean results and from the analysis of variance presented in Table 3.11.b, the serum effect was striking and significant ($p<0,001$), whereas there were no significant differences between the responses of patient and reference lymphocytes when these were cultured in the presence of either patient or control serum. Analysis of these results using non-parametric methods (Friedman's two-way analysis of variance) showed no differences in the statistical significance.

In contrast to serum samples from patients with kwashiorkor, the sera from 10 control children supported the transformation of control cells and reference cells in a normal manner that was quantitatively comparable with that of healthy adult AB serum (Table 3.12.a and Figs. 3.1.c and 3.1.d). Paediatric control cells tended to show a greater incorporation of $[^{14}C]$ than did adult reference lymphocytes and their results were more variable.

Finally, comparison of the results obtained in the two series of experiments indicates that mean $[^{14}C]$ incorporation rates for patient cells and control cells were similar when incubated in reference AB serum (Figs. 3.1.a and 3.1.c), further suggesting that lymphocytes from patients with kwashiorkor were capable of responding normally

FIGURE 3.1

FIGURE 3.1 The effect of kwashiorkor serum on lymphocyte blastogenesis.

Each point in the figures represents the mean $[^{14}C]$ -thymidine incorporated by triplicate cultures of PHA-stimulated lymphocytes cultured in media supplemented with sera indicated at the bottom of each panel. Pairs of points connected by lines represent results obtained when the same lymphocytes were cultured in two different sera in the same experiment. Peripheral blood lymphocytes were isolated from the following subjects:

12 patients with kwashiorkor	(panel a)
10 paediatric control subjects	(panel c)
healthy adults - reference cells -	(panels b and d)

Note that (i) in the presence of kwashiorkor serum lymphocytes obtained from patients with kwashiorkor (panel a) and healthy adults (panel b) responded poorly to PHA-stimulation. In contrast paediatric control serum had no significant effect on the responses of lymphocytes from paediatric control subjects (panel c) and healthy adults (panel d);

(ii) lymphocytes obtained from a few of the patients with kwashiorkor (panel a) responded poorly to PHA stimulation in AB serum but that statistically this was not significantly different from the adult reference lymphocytes (panel b) or paediatric control lymphocytes (panel c) cultured in the presence of AB serum;

(iii) paediatric control lymphocytes tended to show greater incorporation of $[^{14}C]$ than did adult reference cells and their results were more variable.

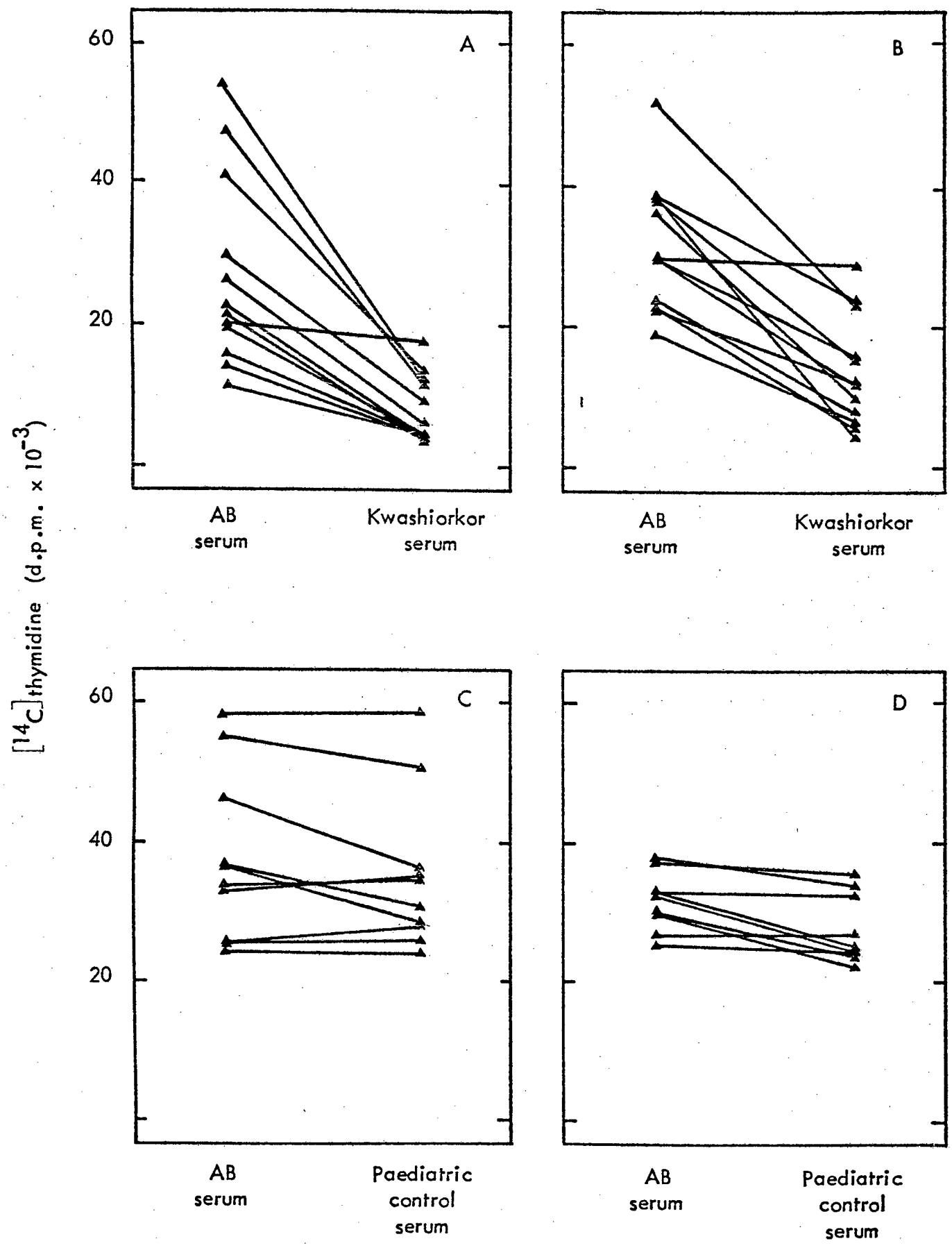

60
40
20
A
AB serum
Kwashiorkor serum
B
AB serum
Kwashiorkor serum
[14C]thymidine (d.p.m. x 10-3)
60
40
20
C
AB serum
Paediatric control serum
D
AB serum
Paediatric control serum

FIGURE 3.1

to PHA provided they were cultured in the presence of normal serum.

It was not always possible, because of the limited numbers of lymphocytes or the volume of blood available, to establish background cultures without PHA stimulation. The results of those cultures that were done are shown in parentheses in Tables 3.11.a and 3.12.a. These results show that spontaneous $[^{14}C]$ -thymidine incorporation is comparable in the various groups with a tendency to slightly higher values in both paediatric groups.

In all 36 patients the effects of kwashiorkor serum were compared with those of AB serum on the $[^{14}C]$ -thymidine incorporation by cultures of normal reference lymphocytes. The results in kwashiorkor serum were expressed as the percentage of the $[^{14}C]$ -thymidine incorporated in PHA-stimulated cultures in normal serum and are shown graphically in Fig. 3.2. The results of similar experiments with paediatric control serum on normal reference lymphocyte cultures are shown alongside for comparison. The results for this larger group of patients are comparable to the results of the 12 patients studied in detail and reported immediately above. The mean value for $[^{14}C]$ -thymidine incorporated by normal reference lymphocyte cultures incubated in kwashiorkor serum from all of the 36 patients was 44% of that obtained with AB serum, as compared to 43% for the 12 patients (Fig. 3.4).

b) Recovery Pattern.

Twelve patients with kwashiorkor were studied at intervals for up to 6 weeks. Clinical improvement of the patients with therapy was accompanied by an increase in the ability of their sera to support PHA-induced lymphocyte transformation. Lymphocyte responses to PHA in reference AB sera and autologous sera were tested at 14 days,

FIGURE 3.2

FIGURE 3.2 The suppressive effect of kwashiorkor serum on the blastogenic responses of normal reference lymphocytes.

This figure compares the effect of kwashiorkor serum (left hand panel) and normal paediatric control serum (right hand panel) on PHA-induced incorporation of $[^{14}C]$ -thymidine by normal adult reference lymphocytes. Each point represents an experiment in which triplicate cultures of reference cells were cultured simultaneously in media supplemented with AB serum and either kwashiorkor serum or paediatric control serum. The mean values of $[^{14}C]$ incorporated in the above cultures were used to calculate the percentage serum effect as follows:-

$$\frac{\text{Reference lymphocytes in subject's serum}}{\text{Reference lymphocytes in AB serum}} \times 100$$

The open bars represent the mean result in each group.

Note that in 36 normal reference lymphocyte cultures the mean kwashiorkor serum effect was 44% and that, with 2 exceptions, the serum effect was lower than the results obtained in the 10 experiments using paediatric control serum.

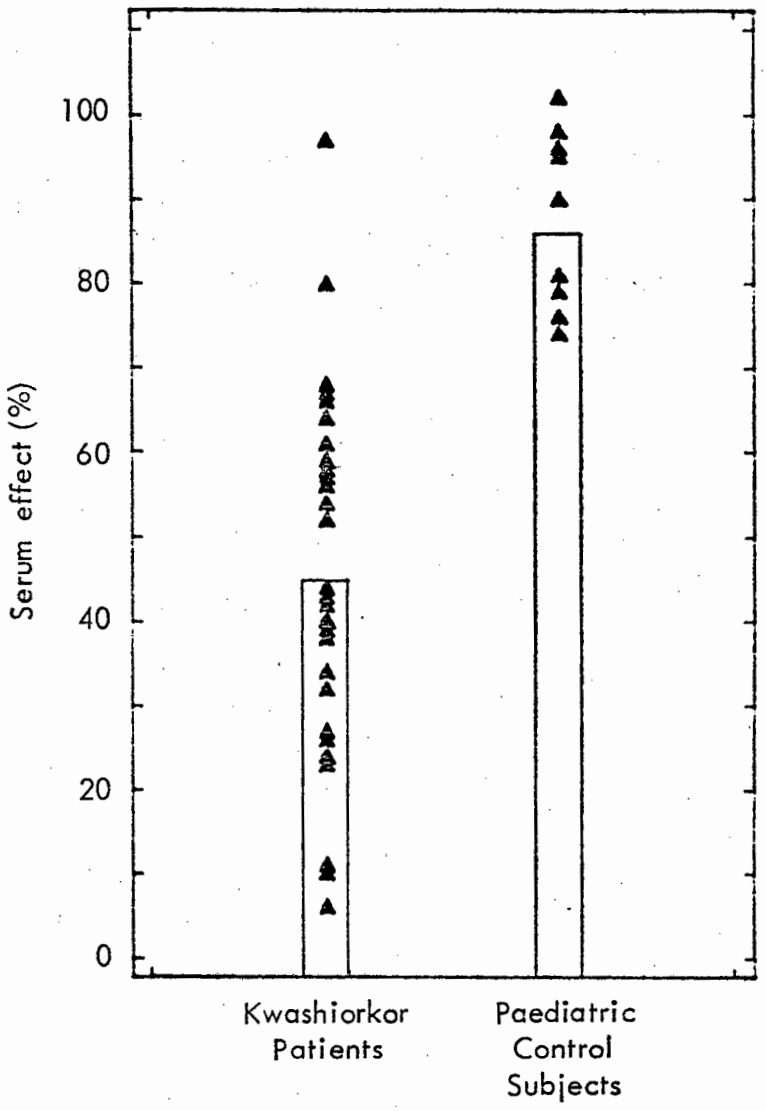


FIGURE 3.2 The suppressive effect of kwashiorkor serum on the blastogenic responses of normal reference lymphocytes.

28 days and 42 days after institution of therapy and the results are summarised in Table 3.13. In Fig. 3.3 the values of $[^{14}C]$ -thymidine incorporated by PHA-stimulated kwashiorkor lymphocytes in autologous serum are expressed as a percentage of the response in AB serum and compared with similarly derived values obtained in control children. By 2 weeks there was a significant increase in the mean value; by 6 weeks all of the 5 patients studied showed considerable improvement in the serum effect, with three of them having values within the control range. When the effects of kwashiorkor serum were compared with AB serum on PHA-stimulated responses of normal reference lymphocytes (Fig. 3.4) the recovery pattern was similar to that obtained when kwashiorkor lymphocytes were used as the responding cells (Fig. 3.3).

The $[^{14}C]$ -thymidine incorporation by cultures of kwashiorkor lymphocytes in AB serum was expressed as a percentage of the value obtained for adult reference lymphocytes simultaneously cultured in AB serum on admission and during the recovery period. These results are depicted in Fig. 3.5 and compared with similarly derived results in the paediatric control patients. Although there was a fairly wide scatter of results in all groups, the mean values were relatively normal. On admission and at 14 days, however, some patients with kwashiorkor did appear to have an intrinsic defect in the ability of their lymphocytes to respond to mitogenic stimulation in normal serum. Lymphocytes from paediatric control subjects and the patients, when tested at 4 and 6 weeks, had a mean responsiveness that was higher than that of adult reference lymphocytes.

TABLE 3.13

PHA STIMULATION OF KWASHIORKOR LYMPHOCYTES IN AB AND AUTOLOGOUS SERUM ON ADMISSION AND AT INTERVALS DURING NUTRITIONAL RECOVERY

($[^{14}C]$ -THYMIDINE INCORPORATION dpm)

	Admission	14 days	28 days	42 days
No. tested	12	9	6	5
Autologous serum				
Mean	7844	18387	17429	28256
(SEM)*	(1404)	(2804)	(3907)	(6553)
AB serum				
Mean	27335	29743	32181	36131
(SEM)	(3952)	(3787)	(2777)	(5384)
T**	7,2	2,1	3,1	0,9
p	<.001	<0.1	<.02	<.4

* SEM = Standard Error of the Mean

** T = Student's T test

FIGURE 3.3

FIGURE 3.3 The suppressive effect of kwashiorkor serum on the blastogenic responses of autologous lymphocytes tested on admission and at intervals during nutritional recovery.

Each point represents an experiment in which triplicate lymphocyte cultures obtained from either kwashiorkor subjects or normal children were stimulated with PHA in the presence of media supplemented with autologous or AB serum. The mean values of $[^{14}C]$ incorporated were used to calculate the percentage serum effect as follows:-

$$\frac{\text{Subject's lymphocytes in autologous serum}}{\text{Subject's lymphocytes in AB serum}} \times 100.$$

Lymphocytes from 12 children with kwashiorkor were studied on admission and, when possible, at 2, 4 and 6 weeks after commencing nutritional therapy, and compared with similarly derived results from 10 normal children. The open bars represent the mean result in each group.

Note the marked serum effect seen on admission and that clinical improvement of the patients with therapy is accompanied by an increase in the ability of their sera to support PHA-induced lymphocyte transformation.

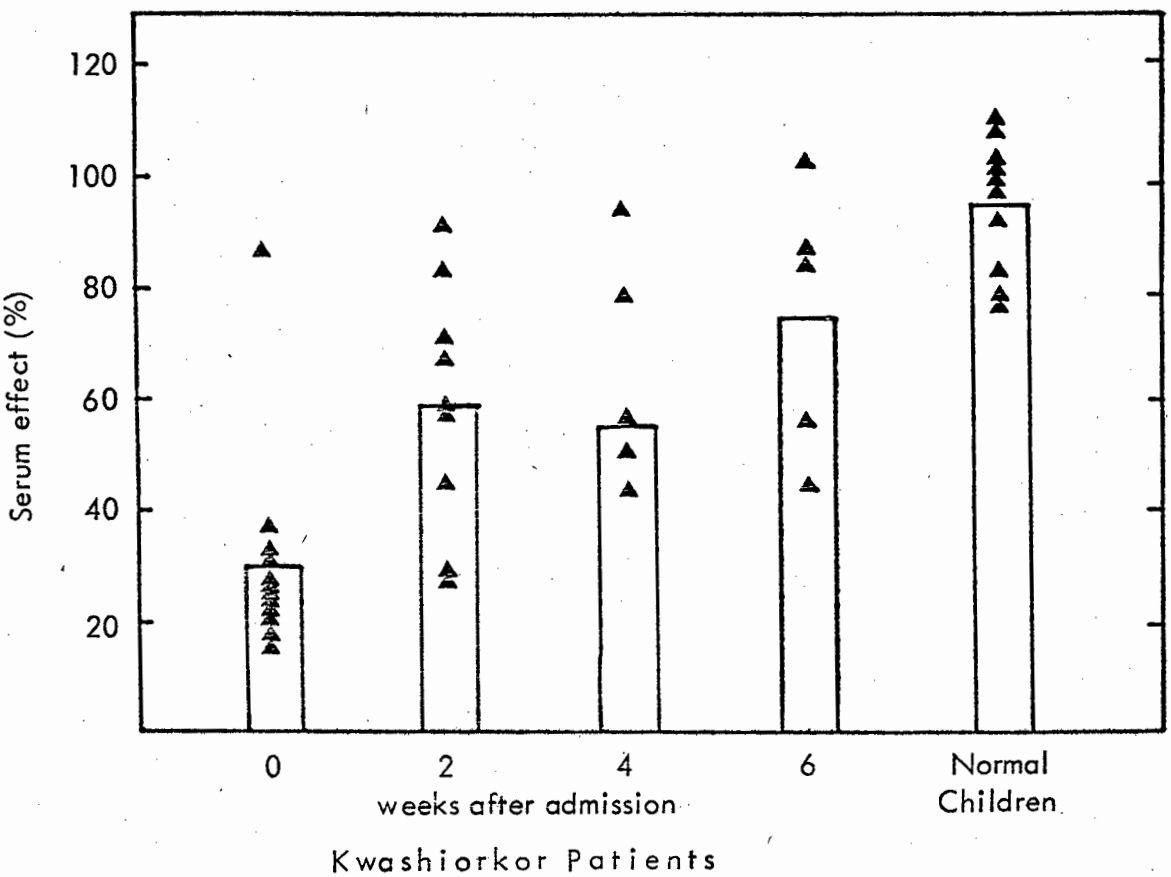


FIGURE 3.3 The suppressive effect of kwashiorkor serum on the blastogenic responses of autologous lymphocytes tested on admission and at intervals during nutritional recovery.

FIGURE 3.4

FIGURE 3.4 The suppressive effect of kwashiorkor serum on the blastogenic response of normal reference lymphocytes tested on admission and at intervals during nutritional recovery.

Each point represents an experiment in which triplicate cultures of normal reference lymphocytes were stimulated with PHA in the presence of media containing AB serum and either kwashiorkor or normal paediatric control serum. The mean values of $[^{14}C]$ incorporation were used to calculate the percentage serum effect as follows:-

$$\frac{\text{Reference lymphocytes in subject's serum}}{\text{Reference lymphocytes in AB serum}} \times 100.$$

Sera from 12 children with kwashiorkor were studied on admission and, when possible, at 2, 4 and 6 weeks after commencing nutritional therapy, and compared with the sera from 10 normal children. The open bars represent the mean result in each group.

Note (i) the marked serum effect seen on admission and the increase in the ability of the kwashiorkor subjects' serum to support PHA-induced lymphocyte transformation that accompanies the patients' clinical improvement;

(ii) the results depicted in the figure are similar to those obtained when the lymphocytes studied were autologous cells (Fig. 3.3).

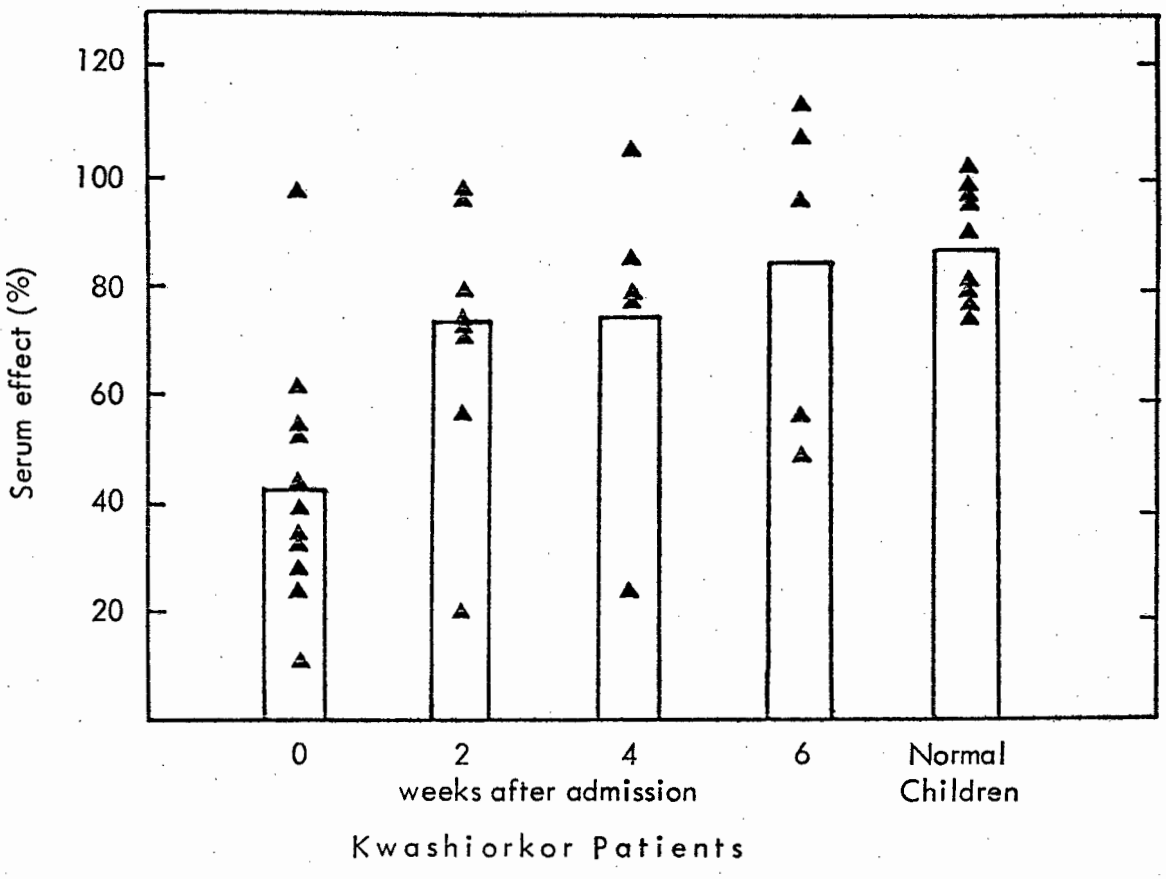


FIGURE 3.4 The suppressive effect of kwashiorkor serum on the blastogenic response of normal reference lymphocytes tested on admission and at intervals during nutritional recovery.

FIGURE 3.5

FIGURE 3.5 The reactivity of kwashiorkor patients' and normal children's lymphocytes to PHA in the presence of media aupplemented with AB serum.

Each point represents an experiment in which cultures of normal reference lymphocytes were compared with lymphocytes from either kwashiorkor patients or normal children. All the cultures were established in triplicate, stimulated with PHA, and supplemented with AB serum. The mean values of $[^{14}C]$ incorporation were used to calculate the percentage reactivity of the subject's lymphocytes as follows:-

$$\frac{\text{Results with subject's lymphocytes}}{\text{Results with reference lymphocytes}} \times 100$$

Lymphocytes from 12 children with kwashiorkor were studied on admission and, when possible, at 2, 4 and 6 weeks after commencing nutritional therapy, and compared with lymphocytes obtained from 10 normal children. The open bars depict the mean result in each group.

Note (i) that, although there is a fairly wide scatter of results in all groups, the lymphocyte reactivity is not significantly different from normal reference lymphocytes;

(ii) on admission and at 2 weeks lymphocytes from some of the patients with kwashiorkor did appear to have an intrinsic defect in their ability to respond to mitogenic stimulation in normal serum;

(iii) lymphocytes from control subjects and from the patients when tested at 4 and 6 weeks had a mean responsiveness that was higher than that of adult reference lymphocytes.

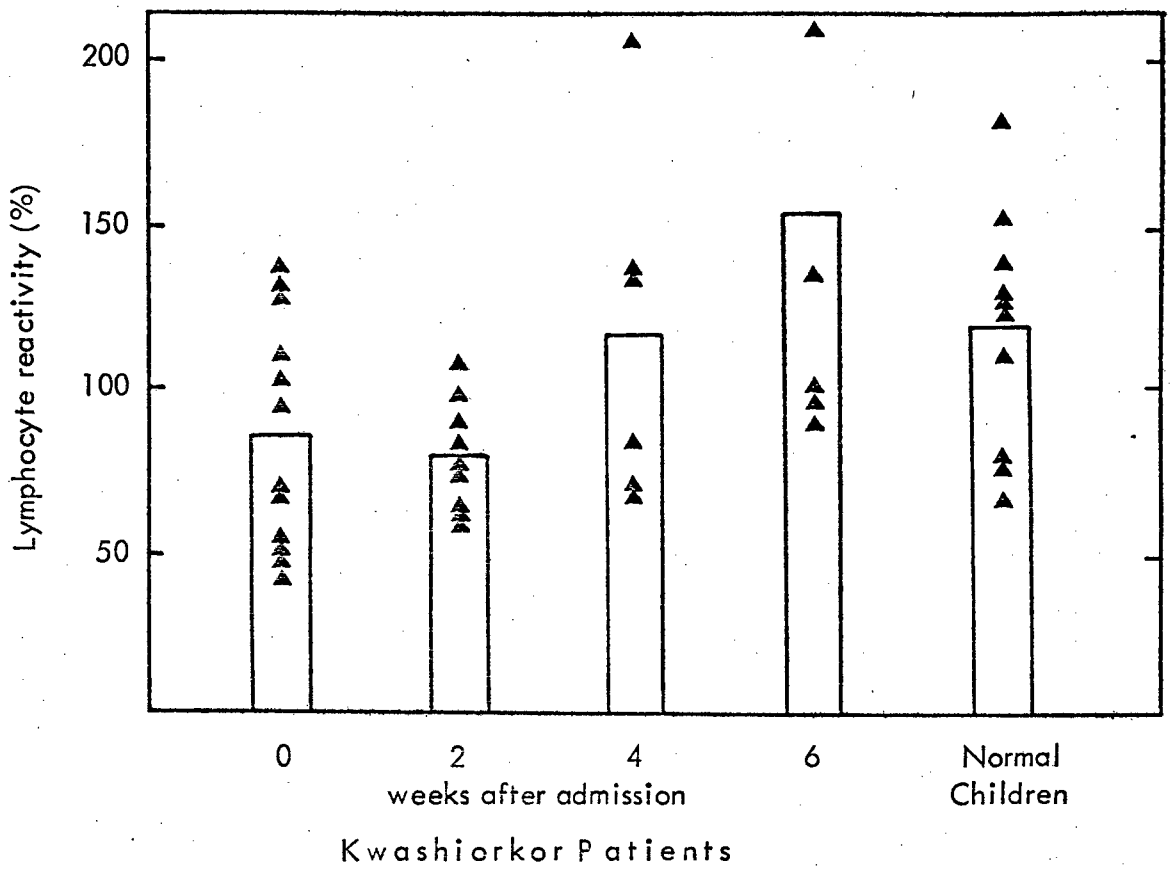


FIGURE 3.5 The reactivity of kwashiorkor patients' and normal children's lymphocytes to PHA in the presence of media supplemented with AB serum.

Mixed Lymphocyte Cultures

a) On Admission.

The difference in the supportive ability of kwashiorkor and AB serum in mixed lymphocyte cultures was examined in 8 patients (Table 3.14 and Fig. 3.6). For one-way cultures one population of lymphocytes was treated with mitomycin C. Patient and reference lymphocytes both responded to and stimulated allogenic cells in a quantitatively similar way when cultured in reference serum whereas, in both one-way cultures with either kwashiorkor or reference lymphocytes as the responder cells and in the two-way cultures, kwashiorkor serum exerted a suppressive effect on $[^{14}C]$ -thymidine incorporation. The effect of kwashiorkor serum was even more striking than that seen in PHA-stimulated cultures. The mean incorporation of $[^{14}C]$ -thymidine in the presence of kwashiorkor serum, when compared with AB serum, was 22% with autologous kwashiorkor responder cells and mitomycin-treated reference lymphocytes as stimulating cells; 16% with reference responder cells and mitomycin-treated kwashiorkor lymphocytes as stimulating cells; and 32% in the two-way cultures. Although the numbers were small, in each of these groups the effect of kwashiorkor serum was significantly different from AB serum ($p < 0.05$; Student's T test).

Also of interest is the observation that, in the presence of AB serum, the results obtained with kwashiorkor lymphocytes as the responder cells were higher than when reference lymphocytes were the responder cells, suggesting the absence of any defect in the capabilities of kwashiorkor lymphocytes to respond in the mixed lymphocyte reaction. A possibility which has not been explored is that kwashiorkor lymphocytes make poor stimulator cells.

TABLE 3.14

MIXED LYMPHOCYTE CULTURES OF LYMPHOCYTES FROM 8 KWASHIORKOR PATIENTS AND NORMAL ADULT DONORS (REFERENCE LYMPHOCYTES) IN KWASHIORKOR AND AB SERUM (dpm $[^{14}C]$ -THYMIDINE INCORPORATION)

Responder lymphocytes	Kwashiorkor	Reference	Kwashiorkor + Reference	Nil
Stimulating lymphocytes (mitomycin-treated)	Reference	Kwashiorkor	No mitomycin	Both mitomycin-treated
AB serum				
Mean (SEM)*	6526 (1628)	5498 (1632)	8785 (1989)	33
Kwashiorkor Serum				
Mean (SEM)	1686 (718)	1234 (550)	3367 (830)	25
% $[^{14}C]$ -thymidine incorporation in kwashiorkor serum	22	16	32	
T**	2,7	2,5	2,5	
p	< .02	< .05	< .05	

* SEM = Standard Error of the Mean

** T = Student's T test

FIGURE 3.6

FIGURE 3.6 The effect of kwashiorkor serum on mixed lymphocyte cultures between kwashiorkor and adult reference lymphocytes.

Mixed lymphocyte cultures were established in triplicate between lymphocytes isolated from patients with kwashiorkor (A) and normal adult reference cells (B). In each experiment points joined by lines represent the mean values of cultures established in media supplemented with either kwashiorkor or AB serum, as indicated at the bottom of the panels. The combinations of lymphocytes shown in the 3 panels were as follows:-

ABm kwashiorkor responder, and reference stimulator lymphocytes;
AmB kwashiorkor stimulator, and reference responder lymphocytes;
AB two-way cultures.

Note that (i) in each combination kwashiorkor serum exerted a significant effect in decreasing the amount of $[^{14}C]$ -thymidine incorporated by cultures;
(ii) kwashiorkor and reference lymphocytes both responded to and stimulated allogeneic cells in a quantitatively similar way when cultured in AB serum.

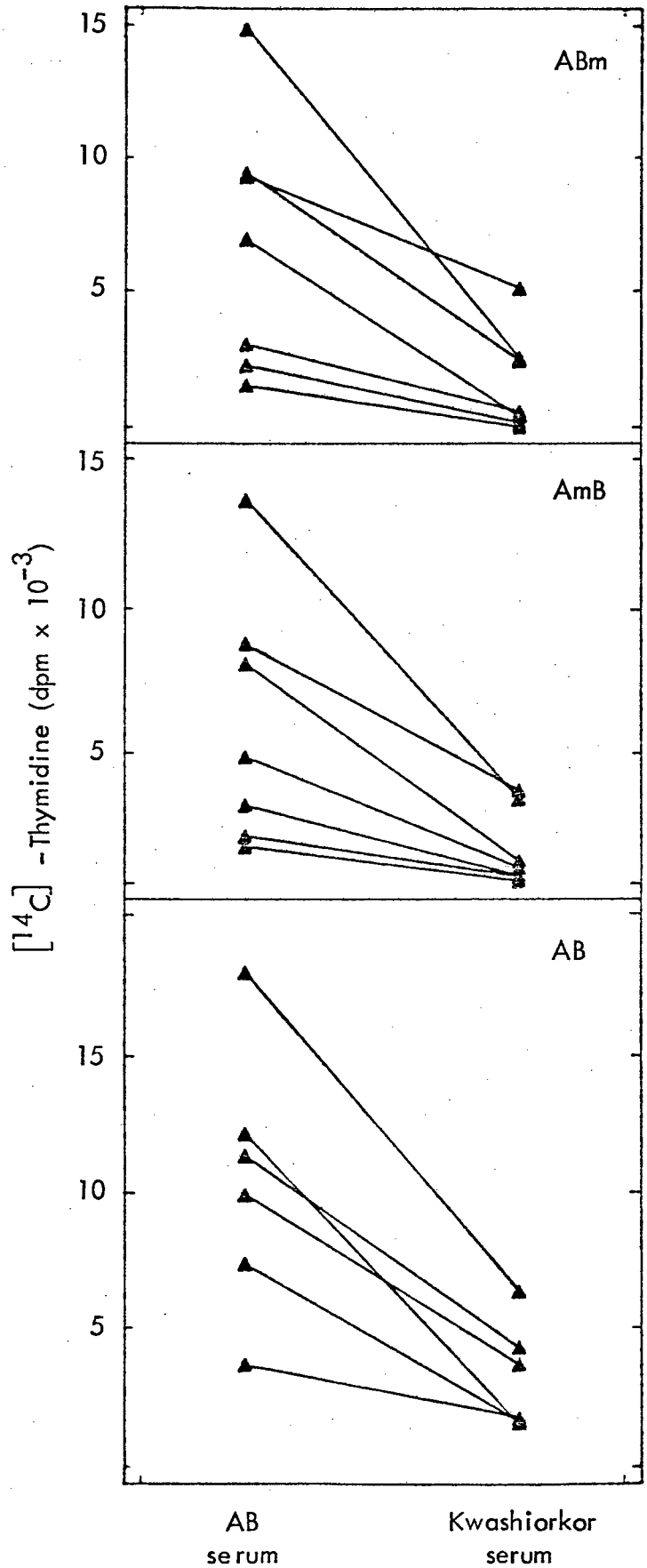

15
10
5
ABm
AmB
AB
[14C] -Thymidine (dpm x 10-3)
AB
serum
Kwashiorkor
serum

FIGURE 3.6

b) Recovery Pattern

Mixed lymphocyte cultures were established at 14 and 28 days in most of these patients. The results are shown graphically in Fig. 3.7, where the values obtained in kwashiorkor serum are expressed as percentages of the corresponding values in reference AB serum. Clinical improvement of the patients with nutritional therapy was accompanied by a recovery in the ability of their sera to support $[^{14}C]$ -thymidine incorporation by mixed lymphocyte cultures. At 28 days, in both one-way mixed lymphocyte cultures in which kwashiorkor lymphocytes were the responder cells, and in two-way cultures, $[^{14}C]$ incorporation by cultures containing kwashiorkor serum was higher than in cultures containing AB serum.

T and B Cell Determinations

T and B lymphocyte numbers in peripheral blood were determined by spontaneous sheep red blood cell rosetting (E rosettes) and rosetting with SRBC linked to antibody and complement (EAC rosettes) for T and B cells respectively in 7 patients with kwashiorkor and simultaneously in 7 adult control subjects.

The results are shown in Fig. 3.8. Although on average the number of lymphocytes forming E rosettes was lower, and the number forming EAC rosettes was higher in the patients with kwashiorkor, there was a fairly wide scatter and the differences were not statistically significant (Student's T test).

Correlation between the Suppressive Effect of Kwashiorkor Serum and the Clinical, Biochemical and Immunological Findings in 36 Patients with Kwashiorkor.

No significant correlation between the suppressive effect of serum from the 36 patients with kwashiorkor on the responses of reference

FIGURE 3.7

FIGURE 3.7 The effect of kwashiorkor serum on the blastogenic response of mixed lymphocyte cultures on admission and at intervals during nutritional recovery.

Mixed lymphocyte cultures between kwashiorkor and normal reference lymphocytes were established on admission and at 2 and 4 weeks after commencing nutritional therapy. The bars represent the mean serum effect seen in the 2 one-way cultures and the two-way culture in the following way:-

 kwashiorkor responder and normal reference stimulator lymphocytes;

 normal reference responder and kwashiorkor stimulator lymphocytes;

 two-way cultures.

Each point represents the effect of kwashiorkor serum when compared with AB serum on simultaneously established cultures and was calculated in the following way:-

$$\text{Serum effect} = \frac{\text{MLC result in kwashiorkor serum}}{\text{MLC result in AB serum}} \times 100$$

Note that, although there is a fairly wide scatter of results in each type of mixed lymphocyte culture, recovery in the ability of kwashiorkor serum to support lymphocyte responsiveness accompanied nutritional treatment at 2 weeks and exceeded the values obtained in AB serum at 4 weeks.

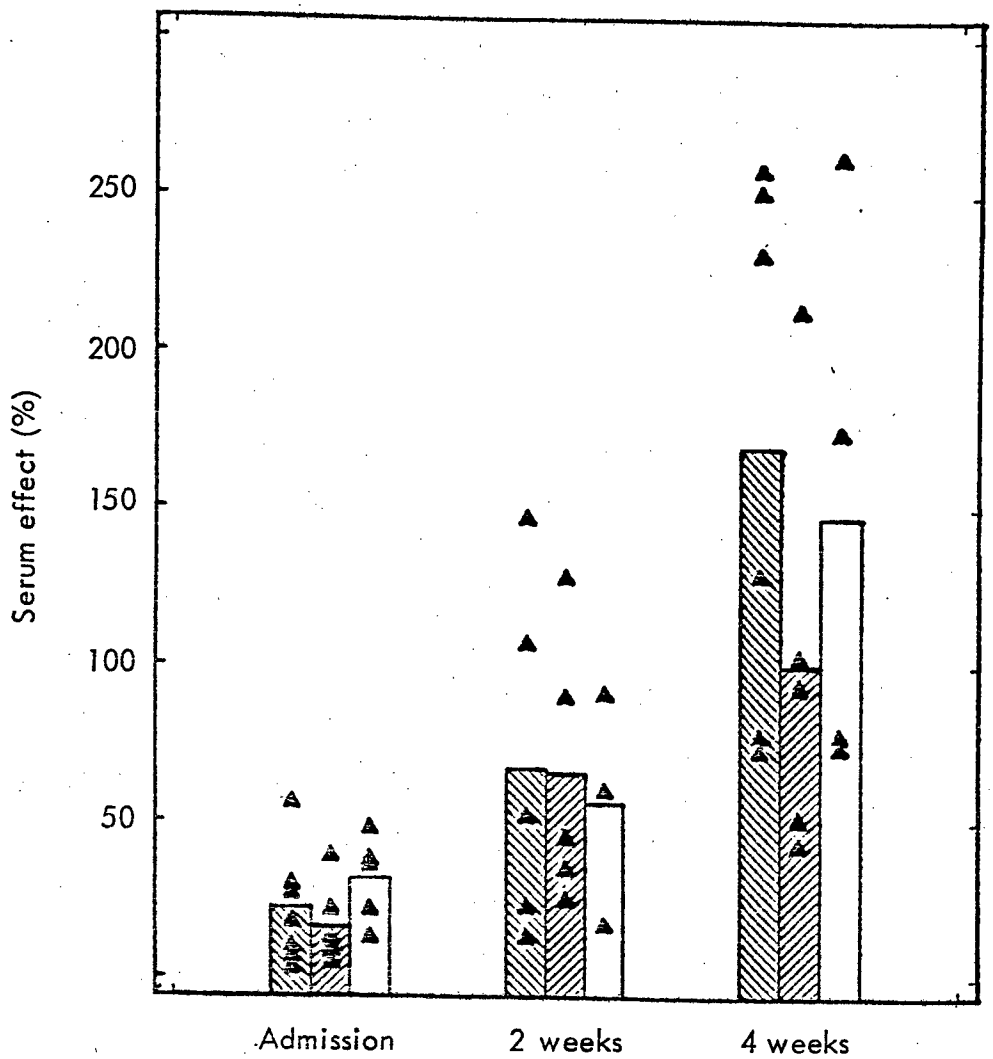


FIGURE 3.7 The effect of kwashiorkor serum on the blastogenic response of mixed lymphocyte cultures on admission and at intervals during nutritional recovery.

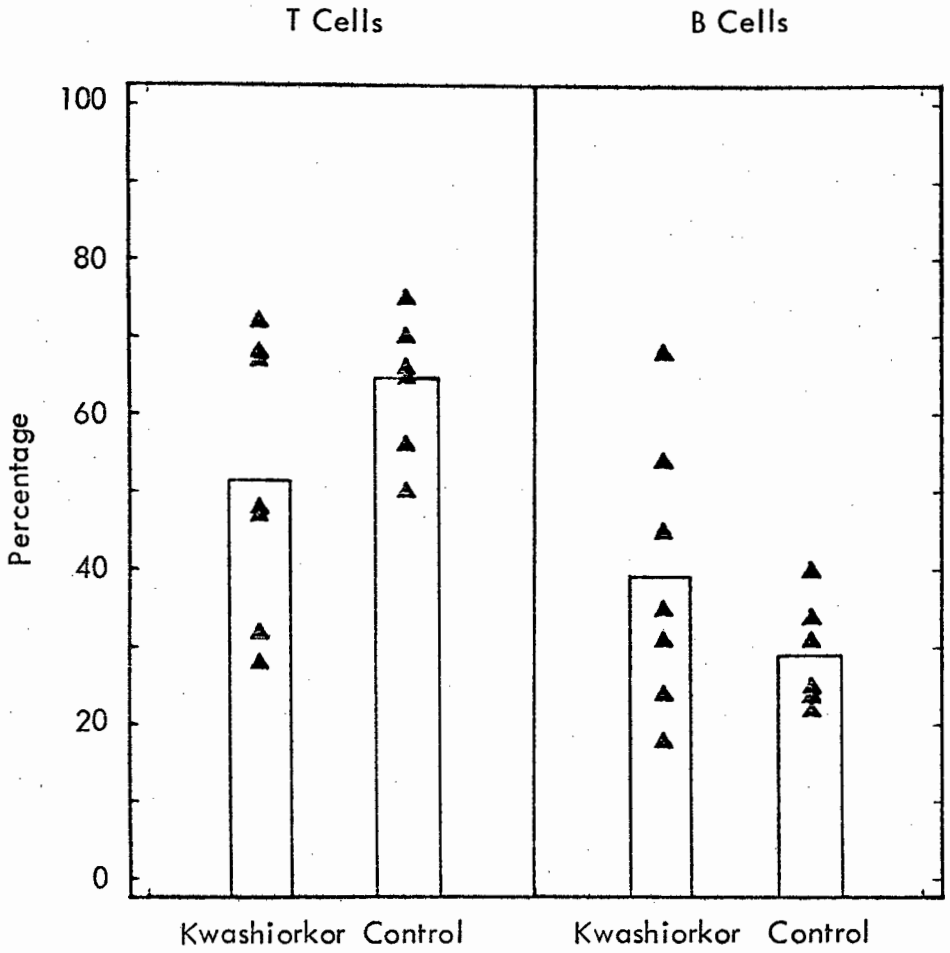


FIGURE 3.8 T and B cell percentages in the peripheral blood of children with kwashiorkor and normal adults.

The points in this figure represent the percentage of purified peripheral blood lymphocytes which formed E rosettes (T cells - left hand panel) and EAC rosettes (B cells - right hand panel). Lymphocytes isolated from patients with kwashiorkor were compared on each occasion with cells from normal adult controls as shown at the bottom of the panels. The height of the open bars represents the mean value in each group.

Note that, although some patients with kwashiorkor have lower proportions of T cells and higher proportions of B cells, the overall mean difference in each case is not great and is not statistically significant.

lymphocytes to PHA, and any of the clinical, biochemical or immunological findings as listed in Table 3.15 was found. Similarly when these findings were compared with the suppressive effect of kwashiorkor serum on autologous lymphocyte responses in a smaller number of patients (19) the results obtained were identical.

The percentage of $[^{14}C]$ -thymidine incorporated in lymphocyte cultures with kwashiorkor serum as compared to AB serum was similar for boys (mean of 52%) and girls (mean of 57%).

Because the majority of children (91%) had some evidence of infection either clinically, radiologically or by bacteriological isolation, it was not possible to establish whether infection influenced the degree of serum suppression.

DISCUSSION

The investigations undertaken in this study were confined to patients suffering from kwashiorkor or marasmic kwashiorkor. These children exhibited the characteristic clinical features of kwashiorkor or marasmic kwashiorkor, namely weight of less than 80% of their expected weight for age, oedema and the typical skin and hair changes found in this condition (Brock and Autret, 1952; Trowell *et al.*, 1954; Waterlow and Alleyne, 1971; Waterlow, 1972). In contrast, the 10 paediatric control children were healthy and well-nourished. Nearly all of the children (33 out of 36) had clinical, radiological or bacteriological evidence of infection. Gopalan (1956) found that 88% of 164 cases of kwashiorkor showed manifest signs of infection. A similar high incidence of infection in kwashiorkor is reported by Pretorius *et al.* (1956) and Scrimshaw *et al.* (1968).

Patients with diseases which in their own right can cause depression of immune responses, such as measles (Coovadia *et al.*, 1974; Sellmeyer

TABLE 3.15

CORRELATIONS BETWEEN THE PERCENTAGE OF [^{14}C] -THYMIDINE INCORPORATED BY PHA-STIMULATED LYMPHOCYTE CULTURES IN KWASHIORKOR SERUM WHEN COMPARED WITH AB SERUM, AND VARIOUS CLINICAL, BIOCHEMICAL AND IMMUNOLOGICAL FINDINGS

Parameter	Number of cases	r	p
Age	36	-0,02	N/S
Percentage expected weight for age	36	0,12	"
Total protein	36	0,21	"
Albumin	36	0,13	"
α_1 globulin	36	0,04	"
α_2 globulin	36	0,15	"
β globulin	36	0,07	"
γ globulin	36	0,22	"
IgG	34	0	"
IgM	34	-0,16	"
IgA	34	-0,10	"
IgE	28	-0,18	"
C3	31	-0,05	"
Lymphocyte count	29	0,29	"
T cells	7	0,68	"
B cells	7	0,52	"
NBT (unstimulated)	14	0,38	"
Anti-A isohaemagglutinin	9	-0,22	"
Anti-B isohaemagglutinin	11	-0,24	"

et al., 1972) and tuberculosis (Harland, 1965; Lloyd, 1968) were excluded from this study.

Serum immunoglobulin levels are usually normal or raised in kwashiorkor (Keet and Thom, 1969; Watson and Freeseman, 1970; McFarlane *et al.*, 1970; Alvardo and Luthinger, 1971; Geefhuysen *et al.*, 1971; Smythe *et al.*, 1971; Chandra, 1972; Neumann *et al.*, 1975a; Suskind *et al.*, 1976). Significantly higher levels of IgA have been found in kwashiorkor by a number of authors (Keet and Thom, 1969; Alvardo and Luthinger, 1971; Suskind *et al.*, 1976). The findings in this study are very similar to those reported by Watson and Freeseman (1970) and Suskind *et al.* (1976).

IgG, IgM, IgA and IgE levels were elevated initially. During treatment a further elevation of IgG, IgM and IgA levels was apparent. However, IgM and IgA levels declined in the latter part of the treatment period but were still elevated at 6 weeks compared with the values obtained in the control subjects.

Antibody production following immunisation in malnourished children has been shown to elicit normal, low or high responses (Pretorius and De Villiers, 1962; Reddy and Srikantia, 1964; Brown and Katz, 1965; Chandra, 1972; El Molla *et al.*, 1973; Coovadia *et al.*, 1974; Neumann *et al.*, 1975; Chandra, 1975a; Suskind *et al.*, 1976). These inconsistencies may be due to the fact that in most cases children were given a high protein diet following immunisation (Scrimshaw *et al.*, 1968) or that different antigens were used (Faulk, Demaeyer and Davies, 1974).

This aspect of immunity has not been explored directly in this study but antibody production to blood group substances (isohaemagglutinin titres) were normal in the children tested. Patients with kwashiorkor have been previously shown to have normal isohaemagglutinin levels

(Kahn, Stein and Zoutendyk, 1957). Although serum immunoglobulin levels are normal or raised and isohaemagglutinin levels are normal, there may well be a deficiency in the ability of children with kwashiorkor to make an adequate antibody response to specific antigens. The further rise in immunoglobulin levels on refeeding suggests that there is an increased capacity for antibody production following treatment.

Low serum C3 levels have been consistently found in kwashiorkor (Smythe *et al.*, 1971; Chandra, 1972; Sirishina *et al.*, 1973; Coovadia *et al.*, 1974; Neumann *et al.*, 1975; Chandra, 1975b). The overshoot of complement levels during nutritional recovery has been shown for C3 and other complement components by Sirishina *et al.* (1973). These authors believe, on the basis of their investigations, that low serum complement levels are due to decreased synthesis of complement rather than increased consumption. This theory is in accordance with the knowledge that kwashiorkor is a deficiency disease. Furthermore, pathological changes in the liver are a prominent feature of kwashiorkor and the liver parenchymal cells are believed to be the principal site of C3 synthesis (Ruddy, Gigli and Austen, 1972).

Several defects in polymorphonuclear cell function have been described in kwashiorkor. Diminished bactericidal capacity of kwashiorkor polymorphonuclear cells has been reported in a number of studies (Selvaraj and Bhat, 1972a; Seth and Chandra, 1972; Douglas and Schopfer, 1974). Other studies have shown normal bactericidal capacity (Arbeter *et al.*, 1971), and Rosen *et al.* (1975) demonstrated that there was no difference between kwashiorkor and control polymorphonuclear function but found lower killing and chemotactic indices when either the kwashiorkor or control subjects had active infections. Phagocytosis by kwashiorkor polymorphonuclear cells has been shown to be normal (Tejada *et al.*, 1964; Douglas and Schopfer,

1974). Derangements in the metabolic activity of polymorphs seem to depend on whether the polymorphs are resting or phagocytosing and on which aspects are investigated. Although hexose-monophosphate shunt activity appears to be normal (Douglas and Schopfer, 1974), NADPH oxidase activity may be decreased (Selveraj and Bhat, 1972b).

Normal nitroblue tetrazolium reduction by polymorphonuclear leukocytes in kwashiorkor patients has been reported by a number of authors (Altay *et al.*, 1972; De Buse, 1974; Rosen *et al.*, 1975). In this study normal NBT reduction confirms the above findings.

There is thus some derangement of certain aspects of phagocytic cell function in kwashiorkor although *in vitro* quantitation is difficult to standardise and interpret. Evidence to support this is the finding of abnormal *in vivo* Rebuck skin window responses in kwashiorkor (Freyre *et al.*, 1973). Defects in serum opsonisation factors may also play a part (Ratnakar and Ramachandraiah, 1974; Seth and Chandra, 1972), although others have claimed that serum factors are not operative (Selvaraj and Bhat, 1972a).

Prominent involution of the thymolymphatic system in children with kwashiorkor has been consistently reported (Vint, 1937; Watts, 1969; Mugerwa, 1971; Smythe *et al.*, 1971; Purtillo and Connor, 1975). A reflection of this involution is the depletion in peripheral blood lymphocytes which may be seen in patients with kwashiorkor. Levels below 2 500 lymphocytes per mm^3 have been noted in 16% (Chandra, 1972) and 35% (Rosen *et al.*, 1975) of children with kwashiorkor. Neumann *et al.* (1975) found that 9% of their patients had levels below 1 000/mm^3. Smythe *et al.* (1971) reported that the peripheral blood lymphocyte count had prognostic significance. There was a 26% mortality in patients with levels below 2 500/mm^3, whereas in those with counts above 2 500/mm^3 only one out of 63 patients died.

Nineteen percent of the patients in this study had peripheral blood lymphocyte counts of less than 2 500/mm^3. Of the three patients who died in this study, two had counts above 2 500/mm^3 but the third child had the lowest peripheral blood lymphocyte count recorded (545/mm^3).

The measurement of cell-mediated immunity by *in vivo* delayed hypersensitivity skin testing has consistently been reported as abnormal in kwashiorkor. Depressed delayed hypersensitivity responses to PPD following BCG vaccination or in cases of active tuberculosis has been widely reported(Harland and Brown, 1965; Harland, 1965; Lloyd, 1968; Chandra, 1972; Abassey *et al.*, 1974). Similar results have been obtained with Candida and other antigens (Geefhuysen *et al.*, 1971; Chandra, 1972; Edelman *et al.*, 1973; Neumann *et al.*, 1975). Following nutritional recovery these skin reactions often became positive. In this study none of the patients responded to PPD antigen initially or after 28 days of treatment. There was no clinical evidence in any patient of tuberculosis and none had received BCG immunisation, therefore lack of prior exposure may account for these results. Initial testing with Candida antigen was negative in all patients tested but after 28 days of treatment 3 out of 7 patients tested gave positive results. These findings are in agreement with those of the authors cited above.

Dinitrochlorobenzene skin sensitisation has been used to examine the capacity of malnourished children to become sensitised and to respond with delayed hypersensitivity reactions to this chemical antigen. Edelman *et al.* (1973) interpreted their results as showing not only a decrease in skin responsiveness but also a defect in sensitisation. A decreased ability to respond to DNCB has been similarly shown in other studies (Smythe *et al.*, 1971; Chandra, 1972; Coovadia *et al.*, 1974; Bang *et al.*, 1975).

DNCB sensitisation and responses in the patients in this study were found to be defective, with only one patient showing an initial response and one further patient responding when rechallenged after 28 days.

Although most published studies of lymphocyte transformation in kwashiorkor have reported diminished cellular responses to mitogenic stimuli in this disease (Table 3.16), the importance of the source of serum used to supplement the lymphocyte cultures has not been emphasized. This factor, together with technical differences in assay procedures, variations in the manner in which results have been interpreted and the use of different diagnostic criteria may have been responsible for some of the discrepancies in the results published.

Histological methods were used to assess lymphocyte responses to PHA stimulation in the reports by Smythe *et al.* (1971), Grace, Armstrong and Smythe (1972) and Geefhuysen *et al.* (1971). In all of these studies cultures were established in autologous serum and low levels of transformation were found, although some of the patients studied by these groups had normal responses.

The results have been more conflicting when the incorporation of radioactive thymidine into lymphocyte DNA has been used to quantitate responses to PHA. Sellmeyer *et al.* (1972) and Schopfer and Douglas (1976) found profoundly diminished responses to PHA in lymphocytes from patients with kwashiorkor. In both studies the lymphocytes were cultured in foetal calf serum (FCS). Moore *et al.* (1974), also using FCS, found abnormal results in only 2 out of 16 children with severe protein-energy malnutrition (PEM) (3 kwashiorkor, 6 marasmic kwashiorkor and 7 marasmus). Neumann *et al.* (1975) found low responses in 4 out of 18 children with kwashiorkor but do not mention the type of serum used to supplement their cultures. Chandra (1972, 1974) reported that all of the patients studied

TABLE 3.16

IN VITRO STUDIES OF CELLULAR IMMUNE FUNCTION IN PROTEIN ENERGY MALNUTRITION (PEM)

Classification of PEM	Lymphocyte Count	Lymphocyte Transformation	Active Serum	E Rosettes	Reference
PEM	14/112 ↓	↓	Auto	-	Smythe *et al* 1971
K	-	12/16 ↓	Albumin	-	Geefhuysen *et al* 1971
K	-	↓	FCS	-	Sellmeyer *et al* 1972
PEM	15/90 ↓	↓	?	-	Chandra 1972
PEM	-	↓	Auto	-	Grace, Armstrong & Smythe, 1972
PEM	-	6/16 ↓	AB	-	Burgess *et al* 1974
PEM	-	↓	Auto	-	Burgess *et al* 1974
K + M	-	N	FCS	-	Moore, Heyworth & Brown 1974
PEM	-	4/4 ↓	AB	-	Coovadia *et al* 1974
PEM	-	↓	AB	↓	Chandra 1974
PEM	N	N	?	↓	Ferguson *et al* 1974
K	N	↓	?	↓	Bhaskaram & Reddy 1974
K	7/20 ↓	-	-	-	Rosen *et al* 1974
M	-	N	?	-	Schlesinger & Stekel 1974
PEM	-	-	-	↓	Bang *et al* 1975
K + M	3/33 ↓	5/27 ↓	?	-	Neumann *et al* 1975
PEM	-	↓	Auto	-	Heyworth, Moore & Brown 1975
K	N	↓	FCS	↓	Schopfer & Douglas 1976

PEM : Protein Energy Malnutrition (unspecified)
K : Kwashiorkor
M : Marasmus
↓ : Decreased
N : Normal
- : Not done

Auto : Autologous serum
AB : Human blood group AB serum
FCS : Foetal calf serum
? : Serum source not specified

by him showed depressed lymphocyte responses to PHA. In the first report the type of serum used in the cultures was not mentioned; in the second study AB serum was used. His patients were classified as having PEM and not all had kwashiorkor. Burgess *et al.* (1974) measured lymphocyte transformation in a group of patients with PEM using both histological and radioactive techniques. They reported a discrepancy in the results obtained with these tests in that, by microscopical evaluation of stimulated cultures, the majority of patients showed a diminished response to PHA, whereas nucleic acid synthesis as measured by the incorporation of $[^{3}H]$-thymidine and $[^{3}H]$-uridine was relatively normal. It should be noted that autologous serum was used to supplement the cultures evaluated histologically, whereas cultures evaluated by isotopic methods were supplemented with AB serum.

Heyworth, Moore and Brown (1975) have reported that heparinised plasma from some children with acute PEM caused marked depression of homologous lymphocyte transformation responses to PHA that did not appear to be due to plasma cytotoxicity. The most severe depression was observed with plasma from patients with kwashiorkor who subsequently died; the effect of plasma from patients with marasmus was minimal.

In this study it was clearly shown that lymphocyte responses to PHA were consistently diminished in cultures supplemented with kwashiorkor sera. This depressed response was seen in both autologous lymphocyte cultures and in cultures of normal reference lymphocytes. Sera from normal children supported the transformation of control cells and reference cells in a normal manner.

This serum abnormality improved with nutritional recovery (Figs. 3.3 and 3.4). The return to normal values was delayed, however, when compared with serum albumin and globulin fractions which had attained normal values after two weeks of treatment (Table 3.5).

The serum effect in the mixed lymphocyte culture experiments was even more striking than that seen in PHA-stimulated cultures. Mixed lymphocyte cultures established in the patients' serum had reached or exceeded the values in AB serum by 4 weeks. Kwashiorkor lymphocytes acting as responder cells gave higher results than when control lymphocytes were responder cells. It may be argued in this case, however, that kwashiorkor lymphocytes make poor stimulator cells, a possibility which has not been explored.

On the basis of a statistical analysis of PHA-induced mitogenic responses in AB serum, intrinsic lymphocyte function in kwashiorkor did not appear to be abnormal. It should be emphasized, however, that this conclusion may well be a matter of interpretation rather than a matter of fact. If, instead of using a comparison between the means and the variances of the results obtained with patient, control and reference lymphocytes to analyse the results, one were to define, from the control results, a "normal" range for thymidine uptake, one might argue that in four cases (cases 5, 6, 9 and 10 of Table 3.11.a) patient cells showed abnormally low responses in AB serum. The results depicted in Fig. 3.5 show graphically that some patients with kwashiorkor had an intrinsically defective lymphocyte responsiveness to PHA in AB serum which returned to normal following nutritional recovery. The data might be interpreted, therefore, as indicating that one third of patients with kwashiorkor showed defective lymphocyte function unrelated to the serum effect. This observation is not contested, particularly in view of the published reports of thymid depletion (Vint, 1937; Watts, 1969; Mugerwa, 1971; Smythe *et al.*, 1971; Purtillo and Connor, 1975) and diminished numbers of peripheral blood T cells as measured by sheep erythrocyte rosetting techniques (Chandra, 1974; Ferguson *et al.*, 1974; Bhaskaram and Reddy, 1974; Bang *et al.*, 1975; Schopfer and Douglas, 1976) in kwashiorkor.

Determination of T and B cell numbers in a small number of patients with kwashiorkor in this study show that 2 out of 7 patients tested had low T cell percentages (Fig. 3.8) although the statistical difference between patients and controls is not significant.

The magnitude of the serum defect in lymphocyte transformation responses showed no correlations with the clinical assessment of nutrition or age. Furthermore, no clearly defined correlation between depressed albumin, α_2 and β globulins, and C3 concentration or with elevated immunoglobulin levels was present to indicate that abnormalities of these serum proteins might be responsible.

CHAPTER 4

DEFICIENCY IN KWASHIORKOR SERUM OF LYMPHOCYTE GROWTH FACTORS

INTRODUCTION

In the previous chapter it was shown that serum from patients with kwashiorkor was deficient in its ability to support lymphocyte transformation *in vitro*. This effect was non-specific in the sense that it was evident with both autologous and normal lymphocytes.

In this chapter experimental results that document in more detail those respects in which kwashiorkor serum fails to provide an adequate medium supplement for *in vitro* lymphocyte transformation are presented.

The experiments were undertaken using normal human lymphocytes isolated from blood specimens collected at the Western Province Blood Transfusion Service.

Kwashiorkor serum for these studies was obtained from 17 of the 36 patients whose results are reported in Chapter 3. In these patients the effects of their serum on homologous lymphocyte responsiveness to PHA and various biochemical parameters were measured as reported in Chapter 3. The balance of the serum was heat-inactivated, sterile filtered and stored at -80^{o}C until used.

Lymphocyte cultures were established in stoppered test tubes or in microtitre plates depending on the nature of each experiment. A detailed description of these methods is outlined in Chapter 2.

STORAGE OF KWASHIORKOR SERUM

Four samples of kwashiorkor serum were stored at -80°C and tested at various time intervals up to three months after separation. Stored sera and fresh sera showed no significant differences in the extent to which they supported (or failed to support!) lymphocyte responses to PHA.

One specimen was deliberately frozen and thawed ten times and then tested. No alteration in its functional properties could be detected.

INTERACTION BETWEEN KWASHIORKOR SERUM, CULTURE MEDIA AND BUFFERS

To define the effects of different buffers and incubation media on the differences between lymphocyte responses in normal and kwashiorkor serum, an experiment was performed in which sera (AB and kwashiorkor), buffers (0,025 M Tris-HCl and 0,015 M Hepes plus 0,025 M sodium bicarbonate) and media (RPMI-1640 and Eagle's MEM) were tested in all factorial combinations. The Hepes-bicarbonate-buffered solutions were incubated in a 5% CO_2 atmosphere. The results of this experiment are presented in Table 4.1. Application of a standard 3-way analysis of variance to these results showed significant ($p < 0,01$) effects of serum (AB > kwashiorkor), medium (RPMI 1640 > Eagle's MEM) and buffer (Hepes-bicarbonate/CO_2 > Tris). No significant pH changes were detected in the cultures containing different sera, buffers or media throughout the incubation period. No significant primary or secondary interactions between the three variables were observed. Since, within the limited range of variables tested, neither buffer nor medium influenced the primary object of the study - i.e. the serum effect - I elected, for reasons of convenience and economy, to perform further studies in Tris-buffered Minimal Essential Eagle's Medium (T-MEM).

TABLE 4.1.a

$[^{14}C]$ THYMIDINE INCORPORATION BY 2 x 10^5 PHA-STIMULATED LYMPHOCYTES IN MICROTITRE PLATES USING DIFFERENT MEDIA, BUFFERS AND SERA

Sera	AB Serum				Kwashiorkor serum			
Media	RPMI 1640		Eagles MEM		RPMI 1640		Eagles MEM	
Buffers	Tris	Hepes bicarb	Tris	Hepes bicarb	Tris	Hepes bicarb	Tris	Hepes bicarb
dpm*	9561	20521	10184	18889	3653	15218	881	11156
(replicate	12296	23291	8584	17235	3613	15107	1307	15367
cultures)	12426	22894	10394	11305	5070	16865	1180	8336
Mean	11428	22235	9721	15809	4112	15730	1123	11620

* dpm = disintegrations per minute

TABLE 4.1.b

THREE-WAY ANALYSIS OF VARIANCE OF DATA FROM TABLE 4.1.a

	Source of variation	Degree of freedom	Sum of squares	Mean squares	F	p
x	serum	1	$2{,}655 \times 10^8$	$2{,}655 \times 10^8$	59,0	< 0,01
y	medium	1	$8{,}701 \times 10^7$	$8{,}701 \times 10^7$	19,4	< 0,01
z	buffer	1	$5{,}707 \times 10^8$	$5{,}707 \times 10^8$	126,9	< 0,01
x.y	serum v. medium	1	$4{,}007 \times 10^5$	$4{,}007 \times 10^5$	0,1	N/S
y.z	medium v. buffer	1	$1{,}279 \times 10^7$	$1{,}279 \times 10^7$	2,8	N/S
z.x	serum v. buffer	1	$1{,}021 \times 10^7$	$1{,}021 \times 10^7$	2,3	N/S
xyz		1	$4{,}853 \times 10^6$	$4{,}854 \times 10^6$	1,1	N/S
remainder		16	$7{,}194 \times 10^7$	$4{,}496 \times 10^6$		

N/S = non-significant

EFFECTS OF MITOGEN AND MITOGEN CONCENTRATION

Mitogen-induced lymphocyte transformation is quantitatively related to the concentration of the mitogenic agent. It may have been argued, therefore, that kwashiorkor serum contained an oligosaccharide or other competitive inhibitor that effectively reduced the concentration of stimulating mitogen. To explore this possibility cells were stimulated with varied concentrations of three mitogens with different specificities: phytohaemagglutinin (PHA), Concanavalin A (Con A) and Pokeweed mitogen (PWM) in the presence of AB serum and kwashiorkor serum. The PHA and Con A cultures were incubated for a total period of 3 days and the PWM-stimulated cultures for 6 days.

As can be seen from the results summarized in Fig. 4.1, lymphocyte blastogenesis was lower in kwashiorkor serum irrespective of the mitogen used, and there was no displacement of the concentration/response curves in kwashiorkor serum. Had there been an inhibitor of mitogen action present in the kwashiorkor serum, one would have expected to find mitogen concentration/response curves shifted to the right with normal responses at higher mitogen concentrations.

THE EFFECT OF KWASHIORKOR SERUM ON THE KINETICS OF RNA AND DNA SYNTHESIS

In order to determine if depressed lymphocyte responses in kwashiorkor serum were due to a delay in the initiation of RNA and DNA synthesis by PHA-stimulated lymphocytes, pulse experiments were performed in which [^{3}H]-uridine (0,5 µCi; specific activity 40 Ci/mmole) or [^{3}H]-thymidine (2,0 µCi; specific activity 2 Ci/mmole) (Radiochemical Centre, Amersham, U.K.) were added to cultures at various times and the cells harvested two hours after the addition of radioactive nucleic acid precursor. The results of these experiments, shown in Fig. 4.2, showed that kwashiorkor serum did not exert its effect by delaying the initiation of either RNA or DNA synthesis.

FIGURE 4.1

FIGURE 4.1 A comparison between the effects of kwashiorkor and AB serum on lymphcyte transformation responses to mitogens at different concentrations.

The blastogenic responses of 2×10^5 normal human lymphocytes cultured in medium supplemented with 20% AB serum (■——■) or 20% kwashiorkor serum (▲——▲). Cells were incubated in the presence of the indicated concentrations of PHA and Con-A for 3 days and of PWM for 6 days. Each point represents the mean $[^{14}C]$ -thymidine incorporated by triplicate cultures and is plotted as a function of mitogen concentration.

Note that lymphocyte blastogenesis was lower in kwashiorkor serum irrespective of the mitogen used and that there is no displacement of the concentration/response curves in kwashiorkor serum.

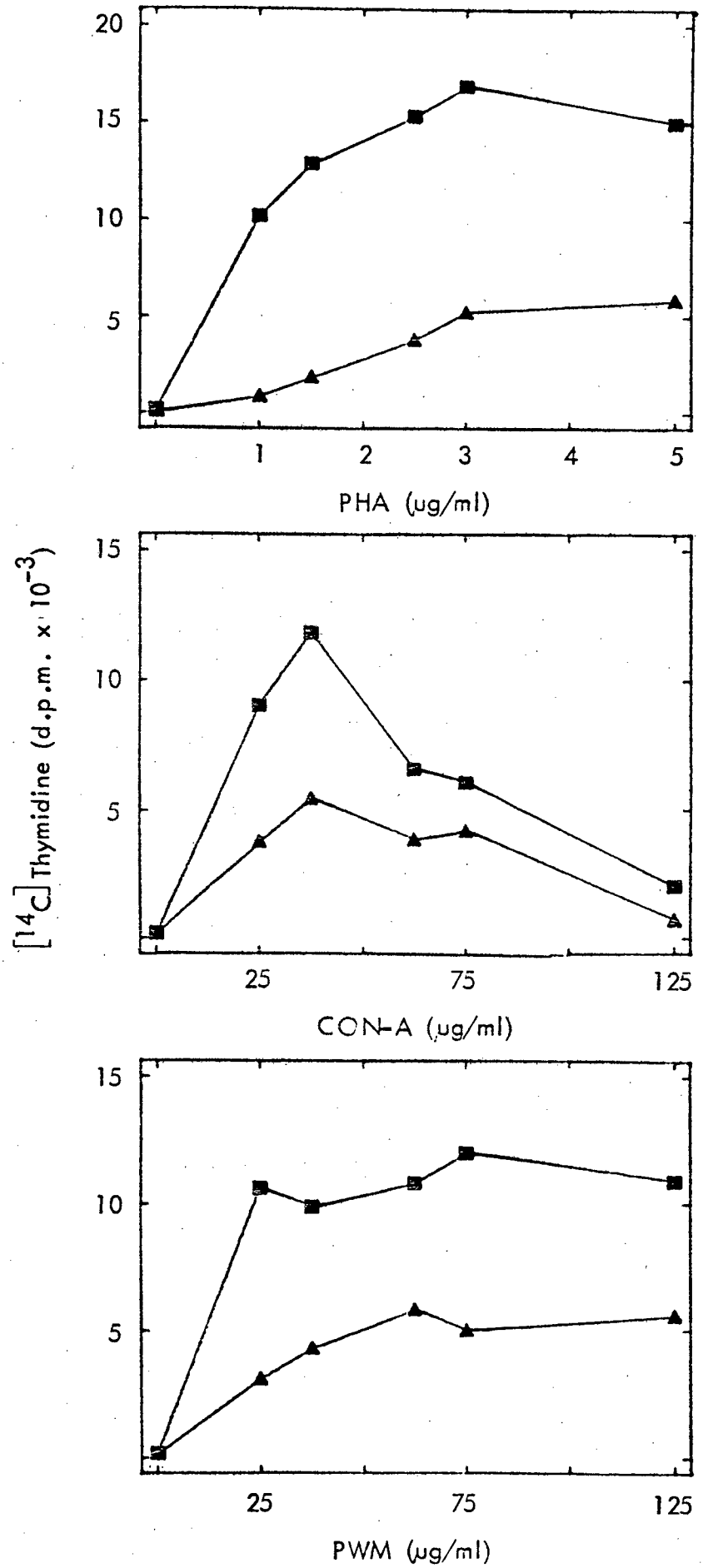

20
15
10
5
1
2
3
4
5
PHA (µg/ml)
[14C] Thymidine (d.p.m. x 10-3)
15
10
5
25
75
125
CON-A (µg/ml)
15
10
5
25
75
125
PWM (µg/ml)

FIGURE 4.1

FIGURE 4.2

FIGURE 4.2 The effect of kwashiorkor serum on the kinetics of RNA and DNA synthesis.

Kinetics of the PHA-induced incorporation of $[^3H]$-thymidine (solid lines) and $[^3H]$-uridine (interrupted lines) into 2 x 10^5 normal peripheral blood lymphocytes cultured in the presence of 20% AB serum (■) or 20% kwashiorkor serum (▲). Radioactive nucleotides were added to triplicate cultures for the final 2 hrs of culture and the mean incorporation of radioactivity was plotted at the time of harvesting as shown.

Note that the onset of DNA synthesis (solid lines) and RNA synthesis (interrupted lines) is not affected by the type of serum used to supplement cultures.

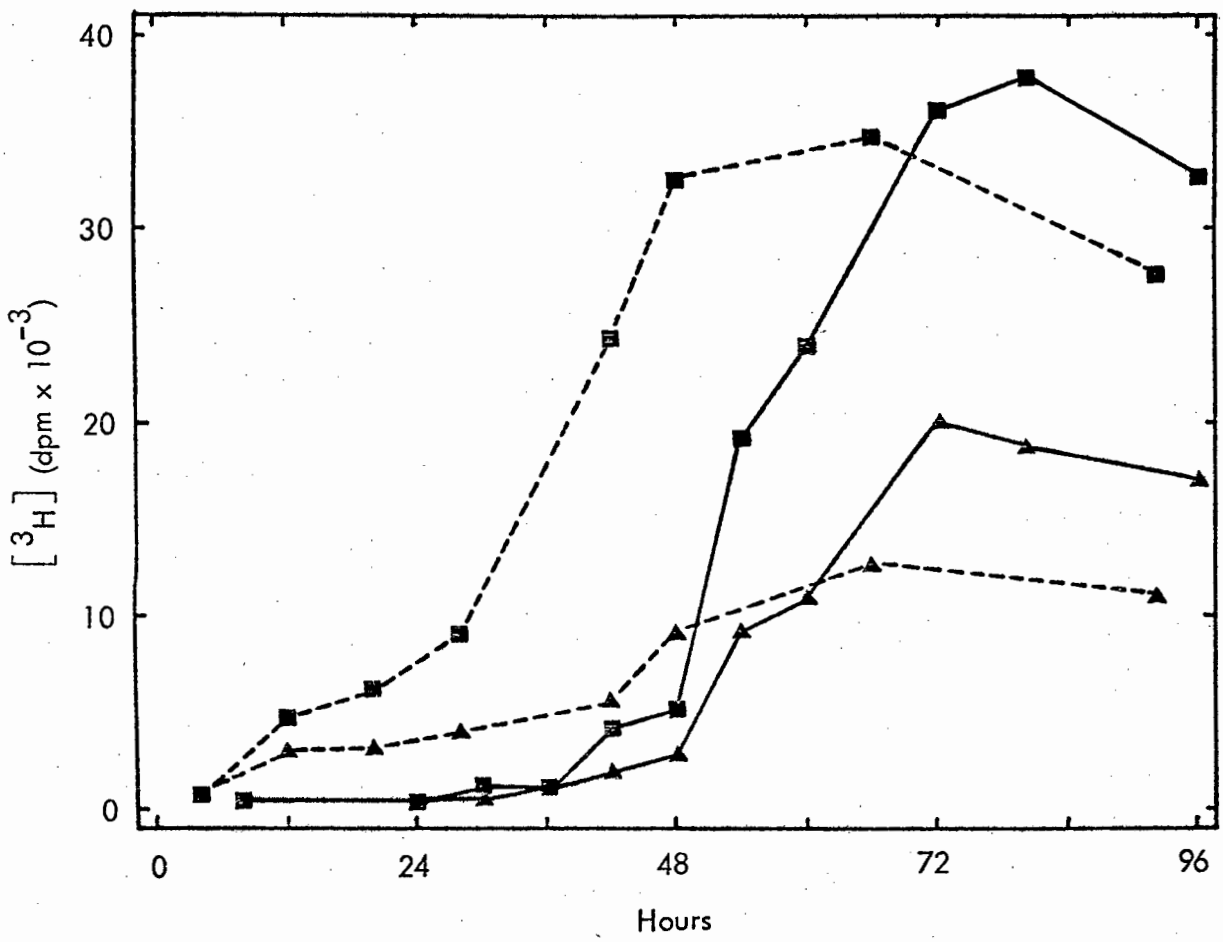


FIGURE 4.2 The effect of kwashiorkor serum on the kinetics of RNA and DNA synthesis.

THE EFFECT OF KWASHIORKOR SERUM ON LYMPHOCYTE VIABILITY AND PROLIFERATION

Trypan blue dye exclusion tests on lymphocytes cultured in normal and kwashiorkor serum showed no differences in the percentage viability of the two populations examined daily during a 5-day culture period. In all cases the percentage viability was greater than 90%.

Nuclear size distribution and total viable cell counts in lymphocyte cultures were obtained using the method of Stewart, Cramer and Steward (1975). One millilitre of a 5 mg/ml solution of Pronase in 0,15 M NaCl was added to the tube cultures. Following incubation at 37°C for 30 min, to digest dead cells, 10 ml of a solution of 3% cetrimide (Kodak, Rochester, U.S.A.) and 1 mM EDTA in 0,14 M NaCl was added; the tubes were agitated vigorously on a Vortex mixer and the released cell nuclei were counted immediately on an electronic particle counter (Coulter Electronics ZBI, Hialeah, U.S.A.), equipped with a multi-channel pulse height analyser for particle sizing (settings: 1/amplitude = 1, 1/aperture = 1/4, lower threshold = 15). This method gave reliable and reproducible viable cell counts and completely overcame the counting difficulties ordinarily associated with agglutination of cells in lectin-stimulated cultures.

Using this method to determine total viable cell counts in PHA-stimulated cultures of 1,3 x 10^6 lymphocytes supplemented with 20% AB serum or 20% kwashiorkor serum, the results depicted in Fig. 4.3 were observed. After an initial drop in numbers of viable cells in both sets of cultures during the first 24 hr, cellular proliferation in the presence of AB serum proceeded more rapidly than in the presence of kwashiorkor serum. Cell numbers in cultures supplemented with AB serum increased progressively from day 1 to reach a density of 1,7 x 10^6 cells/ml on day 6. Cells cultured in the presence of kwashiorkor serum showed a modest proliferation between day 1 and day 3 after which

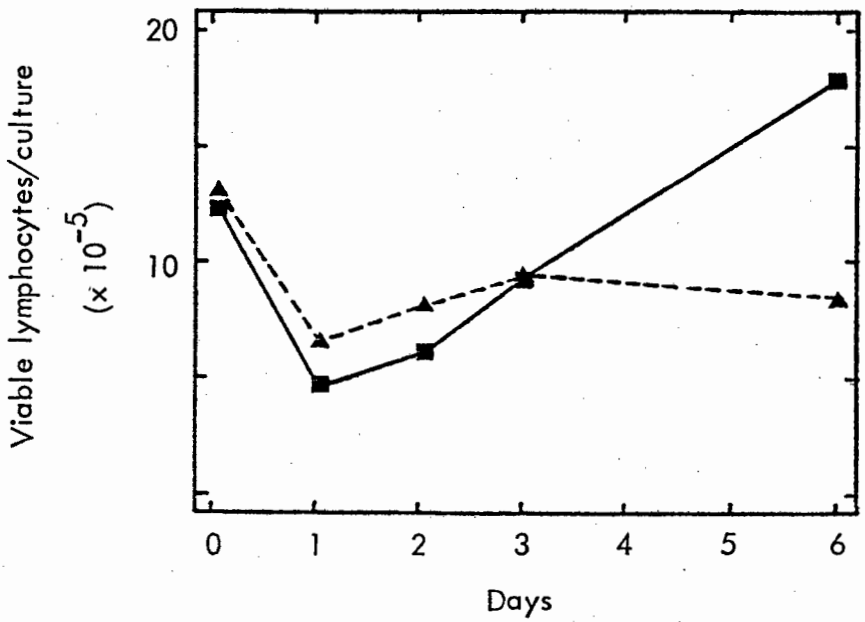


FIGURE 4.3 The effects of kwashiorkor serum on lymphocyte viability and proliferation.

This graph shows the number of viable PHA-stimulated lymphocytes plotted as a function of time in the presence of either 20% AB serum (■——■) or 20% kwashiorkor serum (▲---▲).

Note that, by comparison, the kwashiorkor serum does not appear to exert a cytotoxic effect, but reduces lymphocyte proliferative responses.

cell numbers remained static at 0,8 x 10^6/ml.

Although not entirely conclusive, the Trypan blue exclusion data and cellular kinetic results indicated that inferior lymphocyte responses in kwashiorkor serum were not attributable to a cytotoxic effect. Further evidence to support this conclusion was obtained by culturing 2 x 10^7 lymphocytes in 20 ml of medium containing 50 µg of PHA and 12,5% of either normal AB or kwashiorkor serum. After 40 hrs of incubation the cells were pelleted by gentle centrifugation and the supernatant fluid was carefully removed and replaced with medium containing PHA and either kwashiorkor or AB serum. The cell suspensions were then thoroughly dispersed and 200 µl aliquots dispensed into micro-culture plates. Two-hour pulses of 2 µCi of [^{3}H]-thymidine (specific activity 2 Ci/mmole) were added to triplicate cultures between 40 and 86 hrs and the lymphocyte radioactive thymidine incorporation measured at the times indicated in Fig. 4.4. Removal of kwashiorkor serum at 40 hrs and replacement with AB serum was followed by a gradual increase in DNA synthesis which approached the levels seen in cultures incubated with AB serum throughout, suggesting that the suppression of lymphocyte activation by kwashiorkor serum was reversible and therefore not due to cytotoxic effects.

EFFECT OF KWASHIORKOR SERUM ON BLASTOGENESIS AS MEASURED DIRECTLY

Although incorporation of radioactive thymidine into a population of cultured cells provides an objective and reliable means of quantitating mitogen-responsiveness, it is generally desirable to confirm such results with direct procedures that enumerate cells that have undergone blastic transformation. This was particularly so in the present context, since discrepant results have been obtained with radioactive measurements and blast cell enumeration in studies of

FIGURE 4.4

FIGURE 4.4 The effect of serum change on the kinetics of lymphocyte blastogenesis

Cultures were established in flasks containing 2 x 10^7 lymphocytes and 50 µg of PHA in a final volume of 20 ml of medium supplemented with 12,5% AB serum (AB) or 12,5% kwashiorkor serum (K). After 40 hrs of incubation the cells were pelleted and resuspended in fresh medium containing either kwashiorkor or AB serum to give the sequential combinations indicated by the arrows. The cell suspensions were then distributed in 0,2 ml aliquots in microtitre wells and returned to the incubator. Triplicate microplate cultures were pulsed for 2 hrs with $[^3H]$-thymidine before harvesting at the times shown.

Note that removal of kwashiorkor serum at 40 hrs and replacement with AB serum was followed by a gradual increase in DNA synthesis which approached the levels seen in cultures incubated with the AB serum throughout, suggesting the suppression of lymphocyte activation by kwashiorkor serum was reversible and therefore not due to cytotoxic effects.

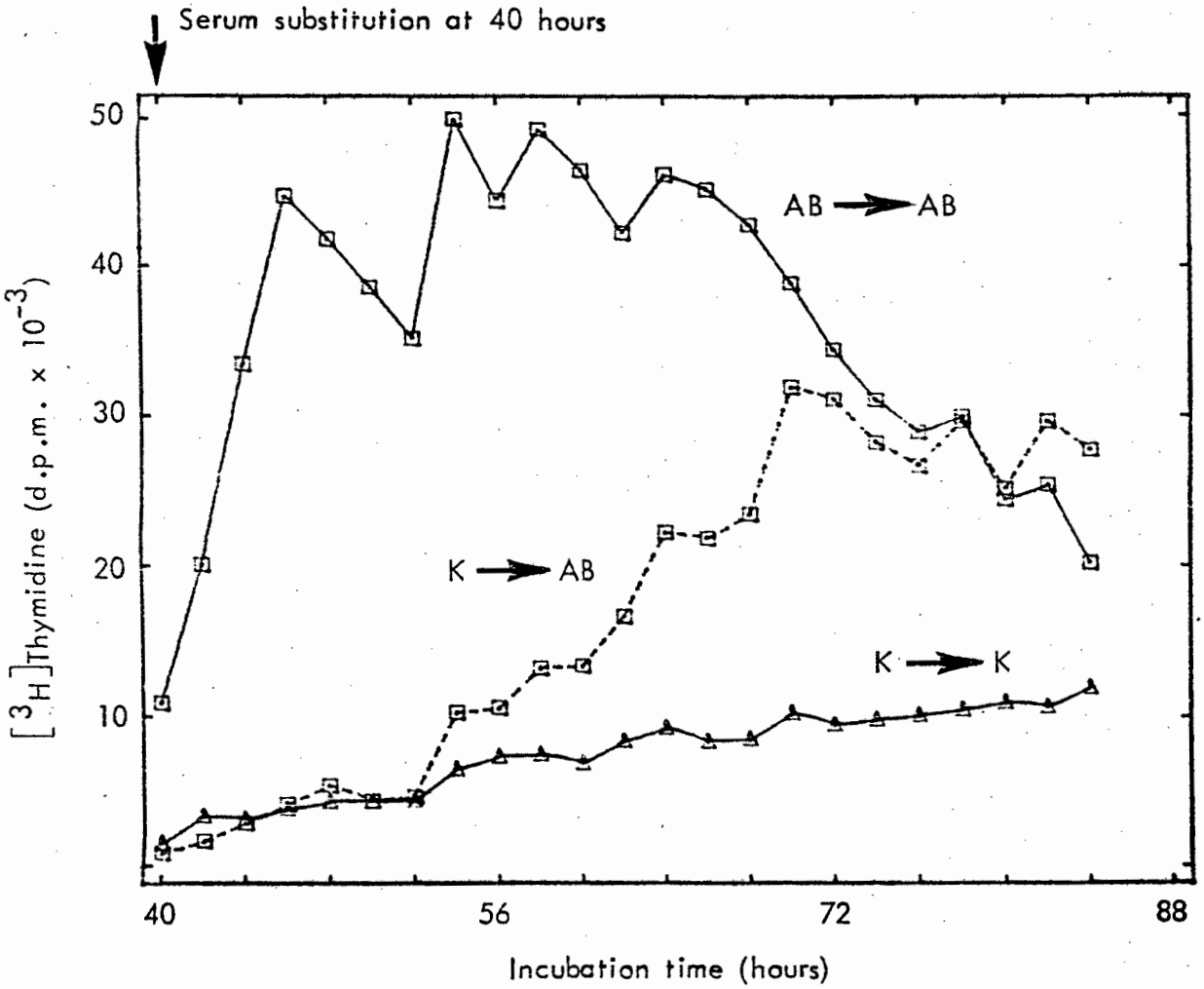


FIGURE 4.4 The effect of serum change on the kinetics of lymphocyte blastogenesis

malnutrition (Burgess *et al.*, 1974).

Two series of experiments were performed, therefore, to document the effect of kwashiorkor serum on the properties of resting cells undergoing transformation as measured by an increase in nuclear size and by autoradiographic techniques.

In the first series, 3-day cultures were treated with pronase and cetrimide and the size distributions of the nuclei so obtained were determined with an electronic particle counter equipped with a multi-channel analyser. As can be seen from a typical experiment summarised in Fig. 4.5, nuclei from unstimulated cells had a relatively narrow size-distribution restricted to channels corresponding to smaller particles. Nuclei from cells stimulated with PHA in AB serum showed a broad, bimodal size-distribution with a distinct fall in numbers of smaller cells and a prominent increase in the numbers of larger cells. Nuclei from cells stimulated with PHA in the presence of kwashiorkor serum showed a size-distribution intermediate between that of unstimulated cells and those treated with PHA in AB serum.

Autoradiography of cells cultured in tubes with 1 μCi of [^{3}H]-thymidine (specific activity 2 Ci/mmole) present for the final 24 hrs of incubation was performed as follows. The cultures were washed twice with medium containing 20% AB serum by gentle pelleting (400 g; 10 min) and resuspension. They were finally resuspended in 0,1 ml of AB serum and streaked, with a fine artist's paint brush, onto clean microscope slides. After drying and fixing for 10 min in methanol, the slides were coated with Kodak NBT2 liquid emulsion containing 1% glycerol and exposed in a light-proof dessicated box at -20^{o}C for 7 days. After developing and fixing the slides were washed with water, dehydrated in alcohol and stained with Giemsa stain. Cells showing 10 or more silver grains over the nuclei were scored as blasts; 200 cells were examined

FIGURE 4.5

FIGURE 4.5 The effect of kwashiorkor serum on lymphocyte nuclei size

This graph shows the size distribution of lymphocyte nuclei after culture in media containing either 20% AB serum without PHA (-------); 20% AB serum with 1 µg/ml of PHA (■——■); or 20% kwashiorkor serum with 1 µg/ml of PHA (▲——▲). After 72 hrs the cells were treated with pronase and cetrimide and the size distribution of the released nuclei determined with an electronic particle counter equipped with a multichannel analyser. Particle volume is directly related to channel number.

Note that i) unstimulated cells have a relatively narrow size distribution restricted to channels corresponding to smaller particles;

ii) nuclei from cells stimulated with PHA in AB serum show a broad bimodal size distribution;

iii) nuclei from cells stimulated with PHA in the presence of kwashiorkor serum show a size distribution intermediate between that of unstimulated cells and those treated with PHA in AB serum.

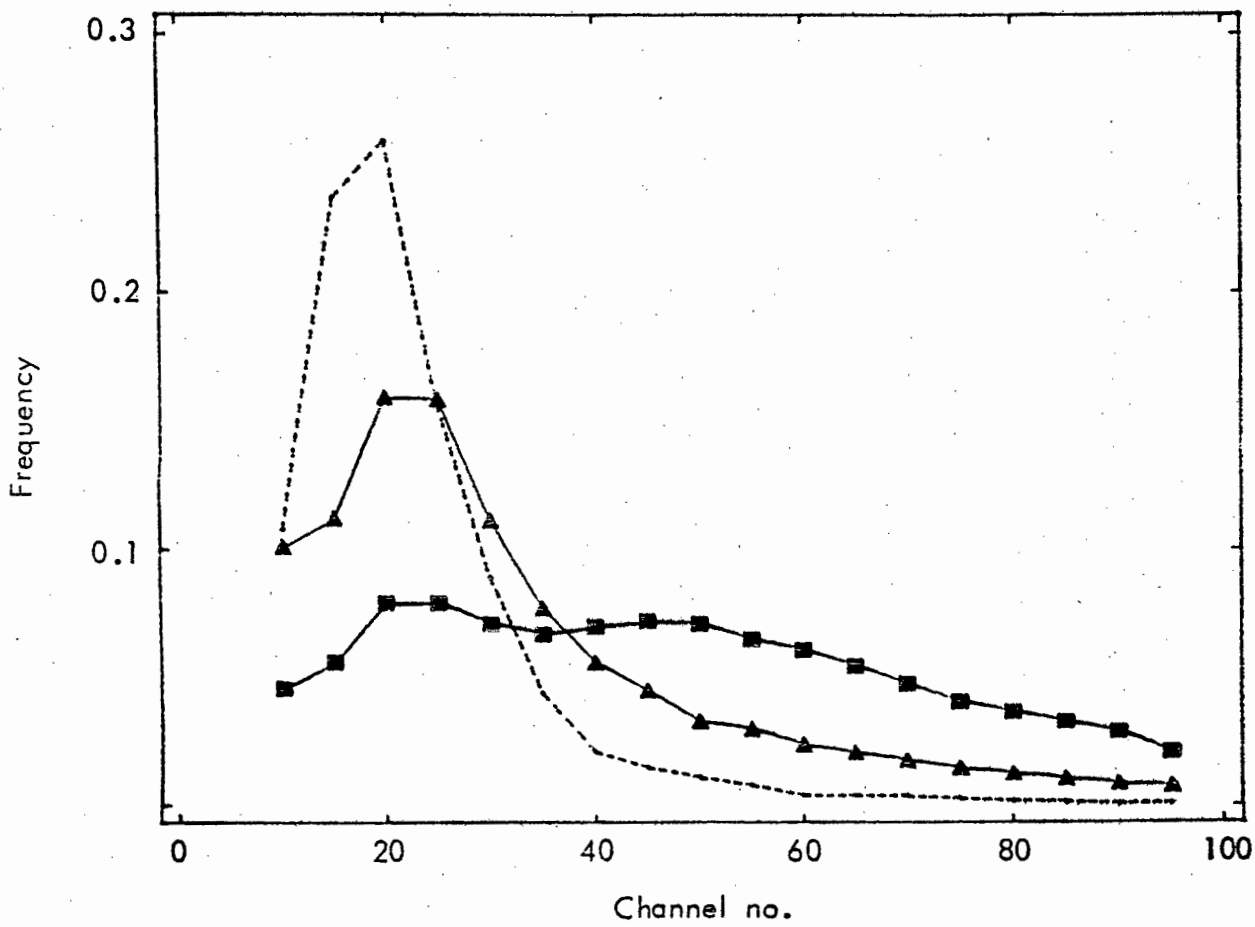


FIGURE 4.5 The effect of kwashiorkor serum on lymphocyte nuclei size

for each culture. A photomicrograph of a typical culture is shown in Fig. 4.6, in which dense accumulation of radioactivity is seen in the nuclei of two of the lymphocytes undergoing blastic transformation.

PHA-stimulated cells cultured with 20% kwashiorkor and AB serum were examined in this way and the results shown in Fig. 4.7 were obtained. Approximately 20% of the cells incubated in AB serum showed nuclear labelling (range 11,5% - 23,5%), whereas less than 10% (range 0,6% - 7,6%) of the cells incubated in kwashiorkor serum underwent blastogenesis by this criterion.

SERUM SWITCH DURING THE MITOGENIC RESPONSE

When the rate of radioactive thymidine incorporation into lymphocyte DNA is measured as a function of time after the addition of mitogen, one finds (Fig. 4.2) that there is a lag period of approximately 48 hrs during which little or no DNA synthesis occurs, followed by a period of rapid incorporation lasting approximately 24 hrs. Although the cellular and biochemical events involved in PHA-induced transformation are not known in any detail, one may infer, from the kinetic experiments, that the mitogenic response involves at least two phases - an early phase during which the responding cells interact with the lectin and proceed through a sequence of more or less synchronous events leading to a later phase of DNA assembly.

The experiment summarised in Fig. 4.8 was performed to see if cells in the early and late phase of the blastogenic response were equally susceptible to the effects of kwashiorkor serum. In this experiment, 2 ml of warmed serum-free medium was added to tube lymphocyte cultures after 24 or 48 hrs of incubation. The cells were pelleted by gentle centrifugation; the medium was removed by careful aspiration; and the cells were resuspended in 1 ml of fresh medium supplemented with

FIGURE 4.6

FIGURE 4.6 Autoradiography of PHA-stimulated lymphocytes cultured in media containing [^{3}H] -thymidine

The photograph shows a typical preparation of lymphocytes cultured in the presence of media containing PHA, AB serum and [^{3}H]-thymidine. The cell suspensions were smeared on glass slides, fixed and exposed with Kodak NBT2 liquid emulsion. After developing and fixing the slides were stained with Giemsa stain.

Note the contrast between the two large lymphocytes undergoing blastic transformation with an accumulation of silver grains over the nuclei and the non-responsive lymphocytes.

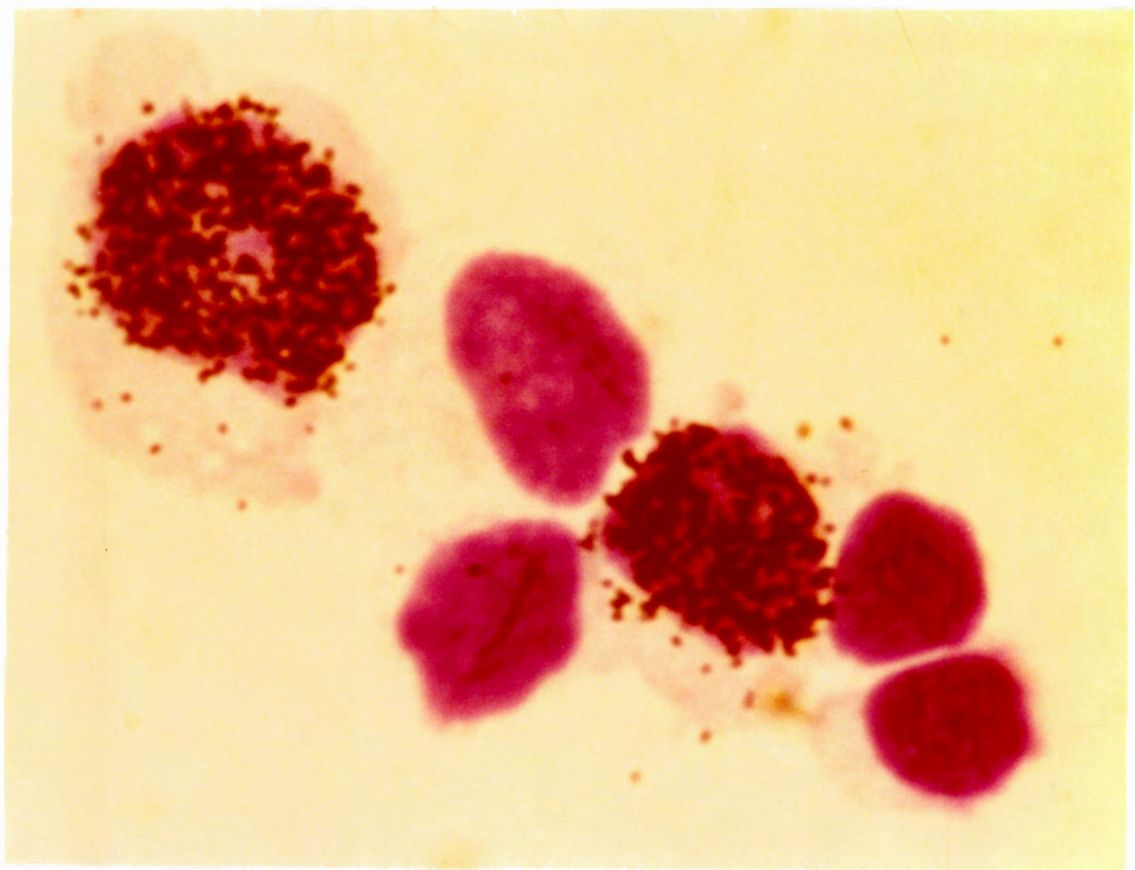


FIGURE 4.6 Autoradiography of PHA-stimulated lymphocytes cultured in media containing [^{3}H] -thymidine

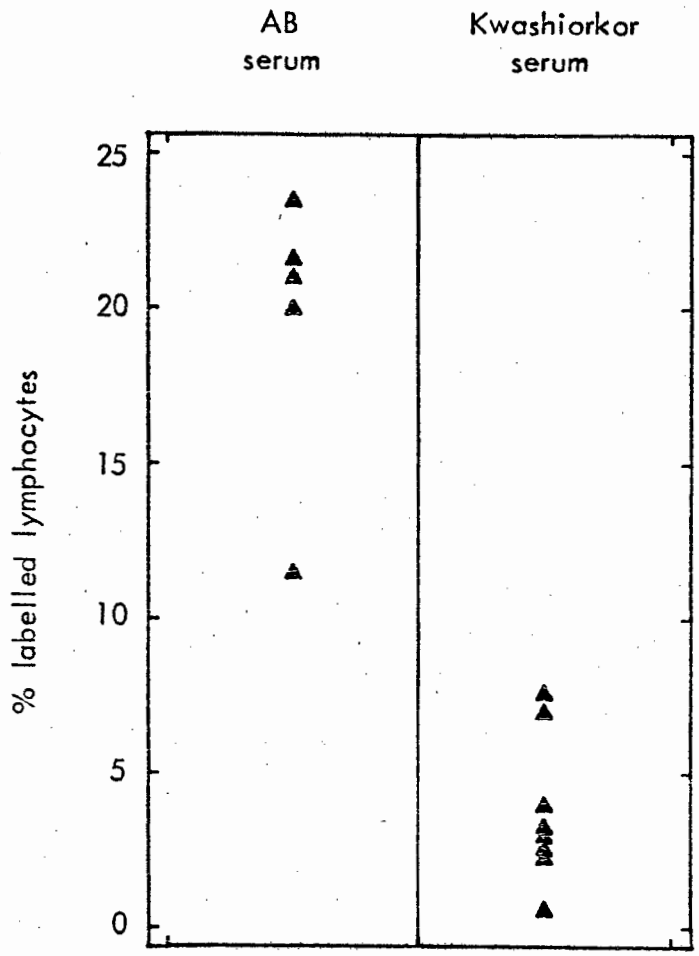


FIGURE 4.7 Blastic transformation of lymphocytes cultured in AB serum and kwashiorkor serum

PHA-induced lymphocyte blastogenesis in media supplemented with 20% AB serum or 20% kwashiorkor serum. Each point represents the results of autoradiographic evaluation of 200 cells from [^{3}H]-thymidine pulsed cultures of lymphocytes obtained from different normal donors. A pool of AB serum and eight different kwashiorkor sera were used to supplement culture media.

Note that in each case normal lymphocytes cultured in kwashiorkor serum showed a markedly decreased percentage of cells undergoing blastic transformation.

FIGURE 4.8

FIGURE 4.8 Serum substitution during the mitogenic response

Serum substitution experiments in which tube cultures of 3×10^5 lymphocytes were stimulated with 1 µg/ml of PHA and incubated in the presence of AB serum (▨) or kwashiorkor serum (□) according to the protocols summarised diagrammatically in the figure. Vertical interruptions in the horizontal bars at 24 or 48 hrs indicate times at which cells were pelleted and resuspended in fresh medium containing either AB or kwashiorkor serum. Radioactive thymidine was added to cultures for the last 24 hrs of incubation and the results shown on the right are the mean values of triplicate cultures.

Note that (i) cells incubated in AB serum throughout incorporated more $[^{14}C]$ -thymidine than those incubated in kwashiorkor serum and that washing and resuspending the cells after 24 or 48 hrs in culture had no significant effect on the control values;

(ii) the effects of kwashiorkor serum were apparent during the early and late phases of the mitogenic response;

(iii) marginally superior responses were encountered when AB serum was present in the first 24 or 48 hrs compared to the last 24 or 48 hrs.

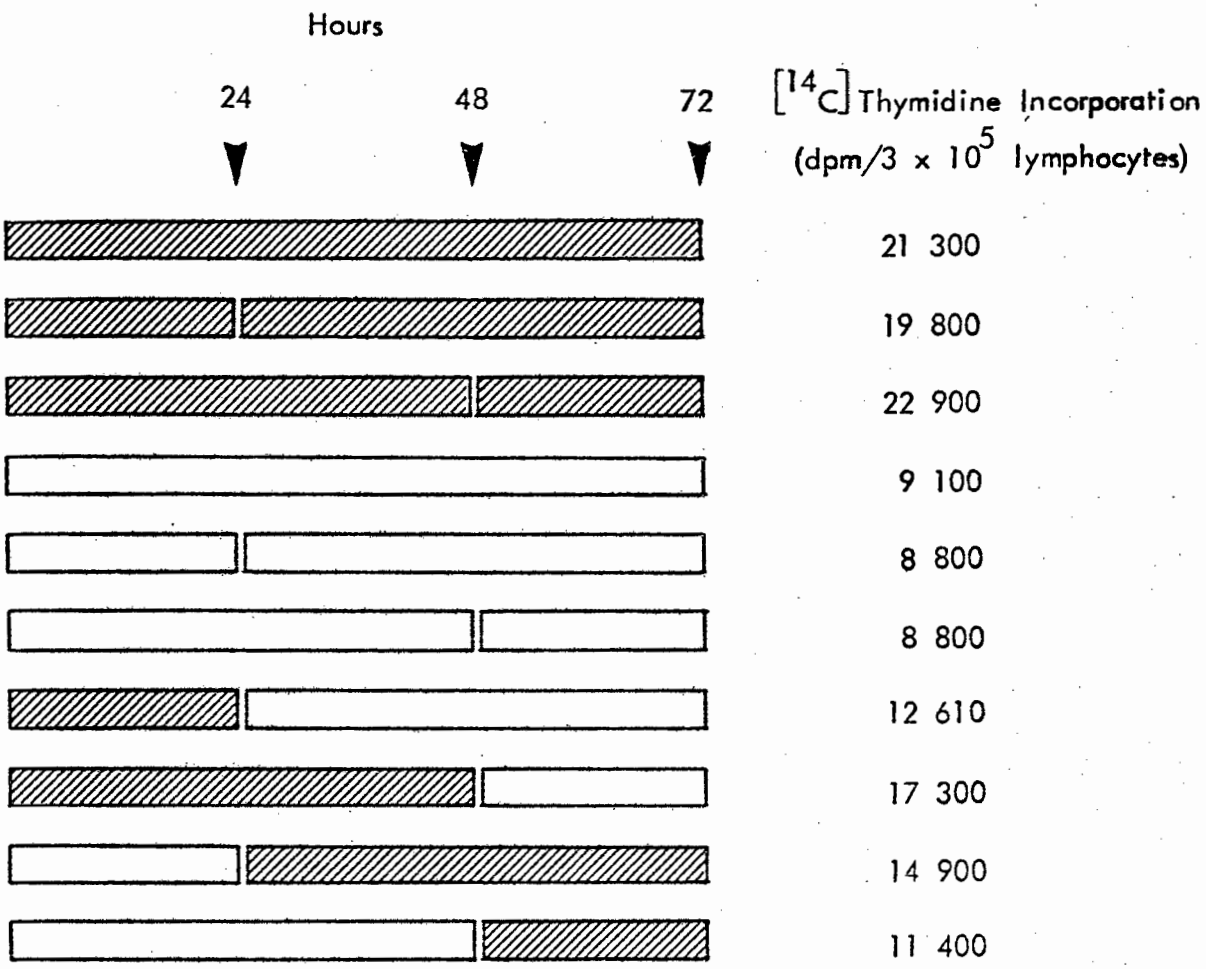


FIGURE 4.8 Serum substitution during the mitogenic response

"switched" serum - i.e. cells that had been incubated initially in kwashiorkor serum were switched to AB serum and *vice versa*. The cultures were then returned to the incubator for the remainder of the 72 hr experimental period. Control cells were either left undisturbed in the same serum throughout or washed without switching the medium. In all cases $[^{14}C]$ -thymidine was present during the last 24 hr period.

Cells incubated in AB serum throughout incorporated approximately 20 000 dpm of $[^{14}C]$ -thymidine; those incubated in kwashiorkor serum incorportaed approximately 9 000 dpm. Washing and resuspending the cells after 24 or 48 hrs in culture had no significant effect on these control values. When AB serum was present during the first 24 hr period, 12 610 dpm were incorporated; when present during the last 24 hr period 11 400 dpm were incorporated. Better responses were seen when AB serum was present during the first 48 hrs (17 300 dpm) or the last 48 hrs (14 900 dpm), but neither of these equalled those seen when AB serum was present throughout.

In conclusion, the effects of kwashiorkor serum were apparent during both the early and the late phases of the mitogenic response. Marginally superior responses encountered when AB serum was present during the first 24 or 48 hrs, compared to the last 24 or 48 hrs, indicated that the cells in the early phase were perhaps more susceptible to the kwashiorkor serum effect than were those in the late phase.

THE EFFECTS OF VARYING CONCENTRATIONS OF AB SERUM, KWASHIORKOR SERUM OR MIXTURES OF THE TWO ON LYMPHOCYTE TRANSFORMATION

The inability of kwashiorkor serum to serve as an adequate medium supplement for optimal blastogenic responses may have been due to the presence of a serum inhibitor or to a deficiency of some essential component present in normal serum. To distinguish between these possibilities,

mixing experiments were performed in which increasing amounts of kwashiorkor serum were added to fixed, limiting concentrations of normal serum.

By choosing concentrations of AB serum that would give thymidine incorporation results falling on the steep portions of the serum/response curve, conditions for the detection of inhibitors in the kwashiorkor serum were optimised.

Results of three such experiments are depicted in Fig. 4.9. When lymphocyte responses to PHA were measured with different concentrations of AB serum in the medium, an approximately sigmoid relationship was observed with a steep slope between 5% and 15% serum and a gradual slope between 15% and 25% serum. Kwashiorkor serum showed a similar relationship but in all cases the lymphocyte responses at any given concentration were lower than those observed with AB serum. When increasing amounts of kwashiorkor serum were added to tubes containing 5% or 7,5% AB serum, lymphocyte responses increased with increasing total serum concentration, albeit at a lower rate than in the tubes when AB serum was added. In no case was a significant fall in thymidine incorporation observed with increasing amounts of kwashiorkor serum. When increasing amounts of normal serum were added to 5% or 7,5% kwashiorkor serum, a curve similar to that obtained with normal AB serum resulted, except that this curve was displaced to the right.

These results indicate that the kwashiorkor sera tested lacked a component present in normal serum required for optimal lymphocyte transformation. They have not been shown to contain inhibitors of blastogenesis.

FIGURE 4.9

FIGURE 4.9 The effects of varying concentrations of AB serum, kwashiorkor serum or mixtures of the two on lymphocyte transformation.

The figure shows the results of three separate experiments (a, b and c) in which sera from different kwashiorkor patients were used to supplement lymphocyte cultures. In each case the graph shows the PHA-induced $[^{14}C]$ -thymidine incorporation into 2 x 10^5 lymphocytes incubated in media supplemented with different concentrations of AB serum (■——■); with different concentrations of kwashiorkor serum (▲——▲); or with a constant, limiting concentration of AB serum to which increasing amounts of kwashiorkor serum were added (△----△). Each point represents the mean value of triplicate cultures.

Note that (i) lymphocyte responses measured with different concentrations of AB serum in the media showed an approximate sigmoid relationship with a steep slope between 5% and 15% serum, and a gradual slope between 15% and 25% serum. Kwashiorkor serum showed a similar relationship but in all cases the lymphocyte response at any given concentration was lower than that observed in AB serum;

(ii) when increasing amounts of AB serum were added to tubes containing either 5% or 7,5% kwashiorkor serum a curve similar to that obtained with normal AB serum resulted, except that this curve was displaced to the right;

(iii) in no case was significant fall in thymidine incorporation observed with increasing amounts of kwashiorkor serum.

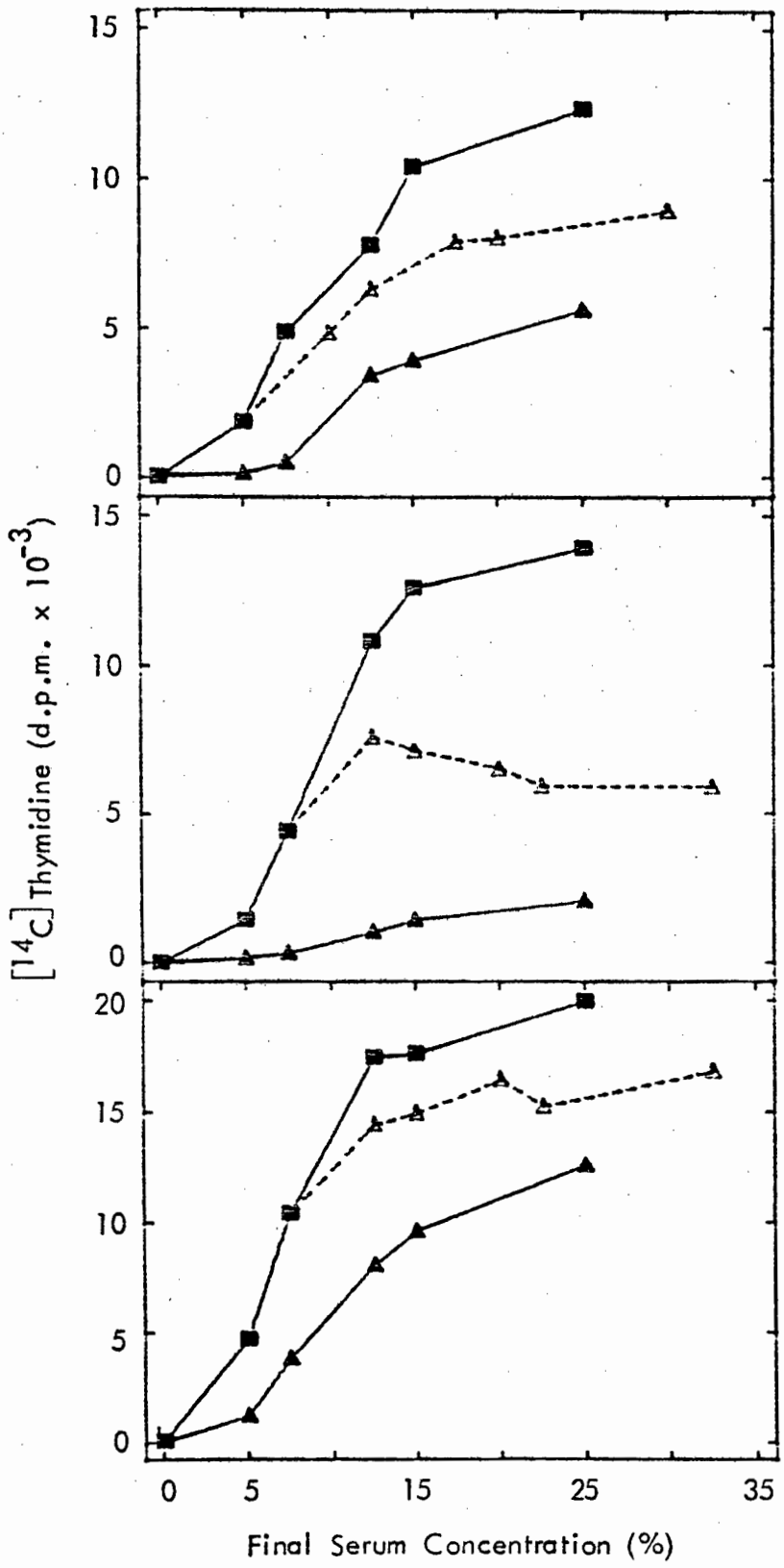


FIGURE 4.9 The effects of varying concentrations of AB serum, kwashiorkor serum or mixtures of the two on lymphocyte transformation.

RECONSTITUTION OF KWASHIORKOR SERUM BY DIALYSIS AND WITH FRACTIONS OF NORMAL SERUM

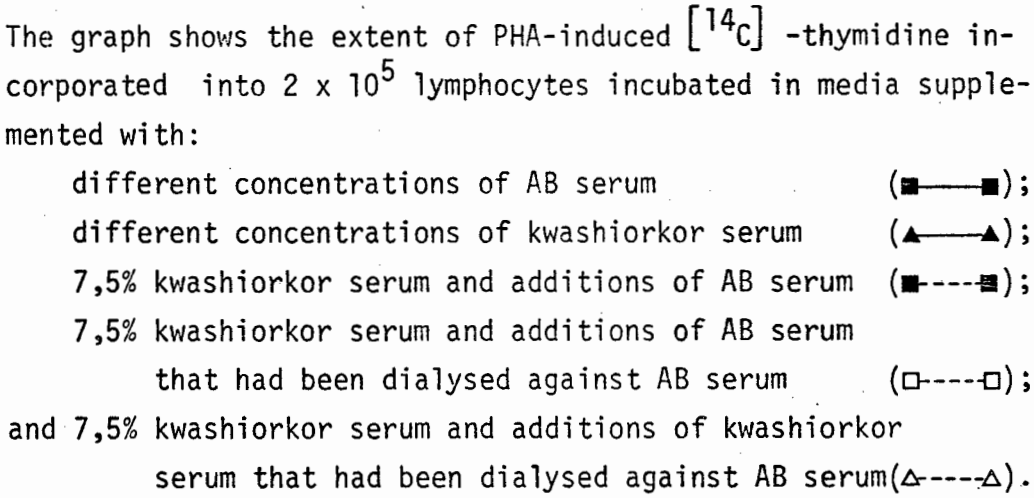
It had been previously observed that dialysis of normal serum against medium completely abolished the ability of this serum to support PHA-stimulated lymphocte transformation. To see whether the poor lymphocyte transformation response seen in kwashiorkor serum was due to the absence of a low molecular weight substance, 1 ml of kwashiorkor serum was dialysed (and, as a control, 1 ml of normal serum) against 40 ml of normal AB serum at $4^{o}C$ overnight.

Increasing volumes of these dialysed sera were added to 7,5% kwashiorkor serum. As can be seen from the results summarised in Fig. 4.10, the dialysed kwashiorkor serum was nearly as effective as AB serum in supporting normal lymphocyte transformation, indicating that kwashiorkor serum was relatively deficient in some low molecular weight dialysable component(s).

The low molecular weight of the restoring AB serum fraction was confirmed in an experiment in which a cellophane membrane ultrafiltrate of AB serum was added, in increasing amounts, to cultures containing 7,5% kwashiorkor serum. The results, with those of appropriate control cultures, are given in Fig. 4.11. As can be seen, addition of increasing amounts of AB ultrafiltrate to cultures containing 7,5% kwashiorkor serum progressively augmented lymphocyte transformation responses. Cultures containing 7,5% kwashiorkor serum and ultrafiltrate equivalent to 25% AB serum showed thymidine incorporation values that were higher than those observed with optimal concentrations of AB serum. Essentially similar results were observed (Fig. 4.12) when an ultrafiltrate of AB serum was prepared with an Amicon UMO 5 membrane (Amicon Corporation,

FIGURE 4.10

FIGURE 4.10 Reconstitution of kwashiorkor serum by dialysis against normal serum



The graph shows the extent of PHA-induced $[^{14}C]$ -thymidine incorporated into 2 x 10^5 lymphocytes incubated in media supplemented with:

- different concentrations of AB serum (■——■);
- different concentrations of kwashiorkor serum (▲——▲);
- 7,5% kwashiorkor serum and additions of AB serum (■----■);
- 7,5% kwashiorkor serum and additions of AB serum that had been dialysed against AB serum (□----□);
- and 7,5% kwashiorkor serum and additions of kwashiorkor serum that had been dialysed against AB serum (△----△).

Each point represents the mean result of triplicate cultures.

Note the similarities between the curves obtained when 7,5% kwashiorkor serum was supplemented with either AB serum, AB serum dialysed against AB serum and kwashiorkor serum dialysed against AB serum.

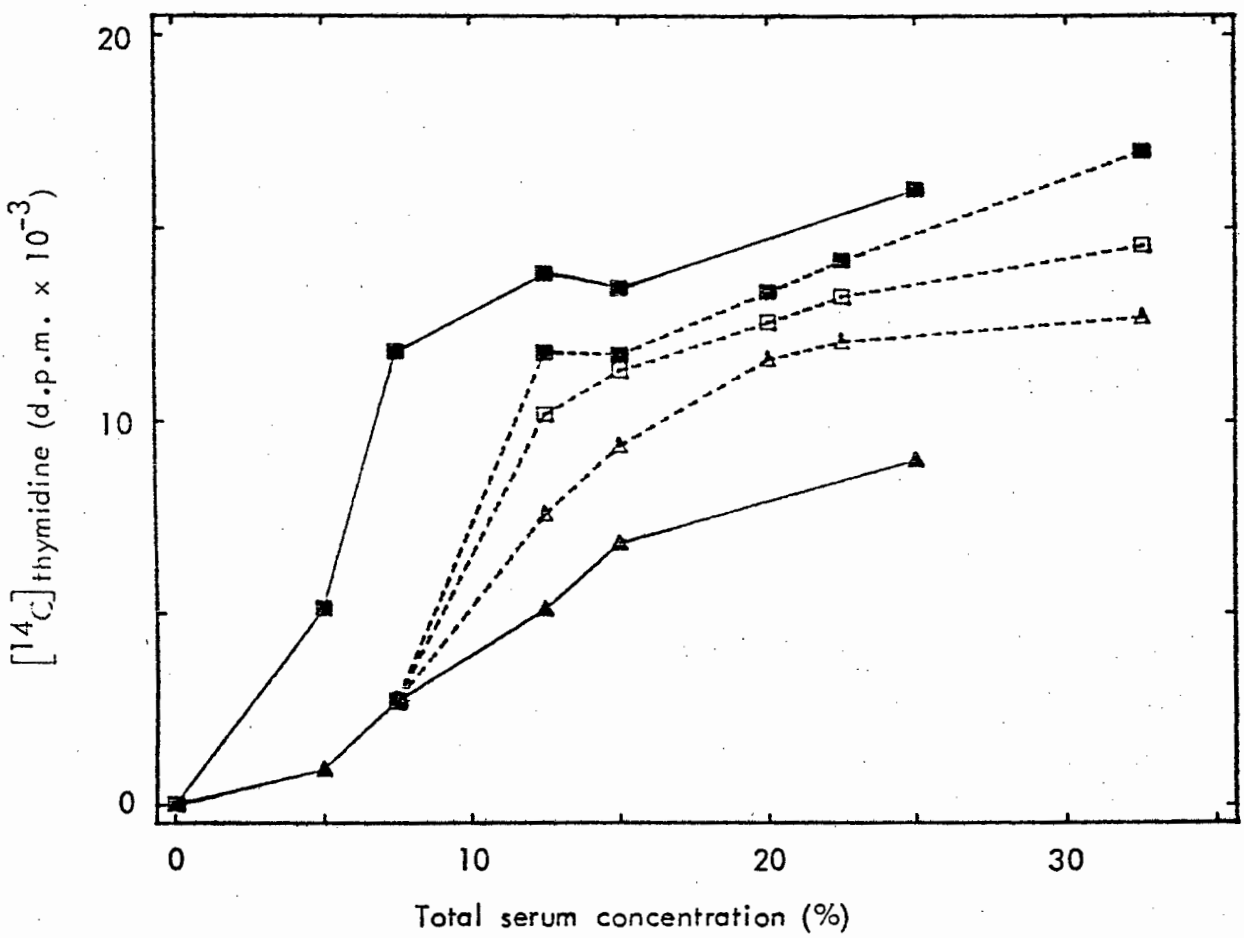
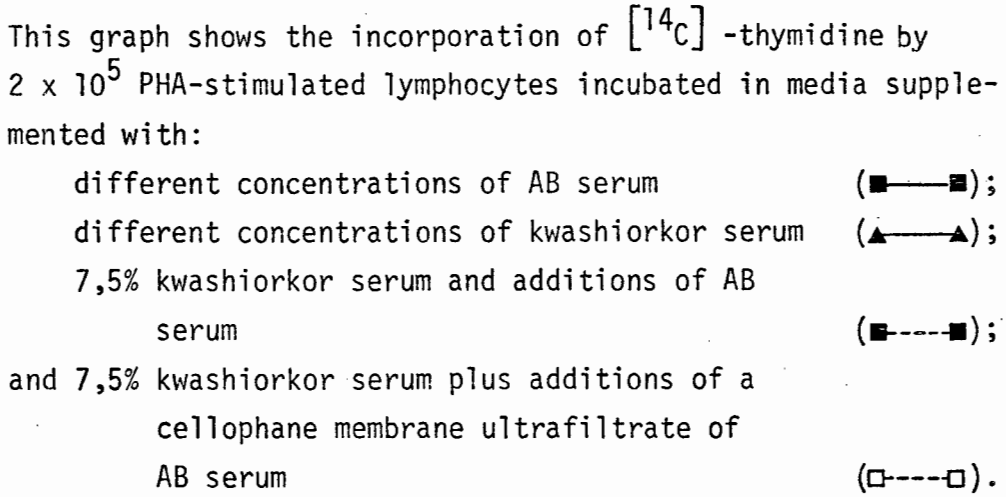


FIGURE 4.10 Reconstitution of kwashiorkor serum by dialysis against normal serum.

FIGURE 4.11

FIGURE 4.11 Reconstitution of kwashiorkor serum by an ultrafiltrate of normal serum



This graph shows the incorporation of $[^{14}C]$ -thymidine by 2×10^5 PHA-stimulated lymphocytes incubated in media supplemented with:

- different concentrations of AB serum (■——■);
- different concentrations of kwashiorkor serum (▲——▲);
- 7,5% kwashiorkor serum and additions of AB serum (■----■);

and 7,5% kwashiorkor serum plus additions of a cellophane membrane ultrafiltrate of AB serum (□----□).

The points shown are the mean results of triplicate cultures.

Note that the addition of increasing amounts of AB ultrafiltrate to cultures containing 7,5% kwashiorkor serum progressively augmented lymphocyte transformation responses.

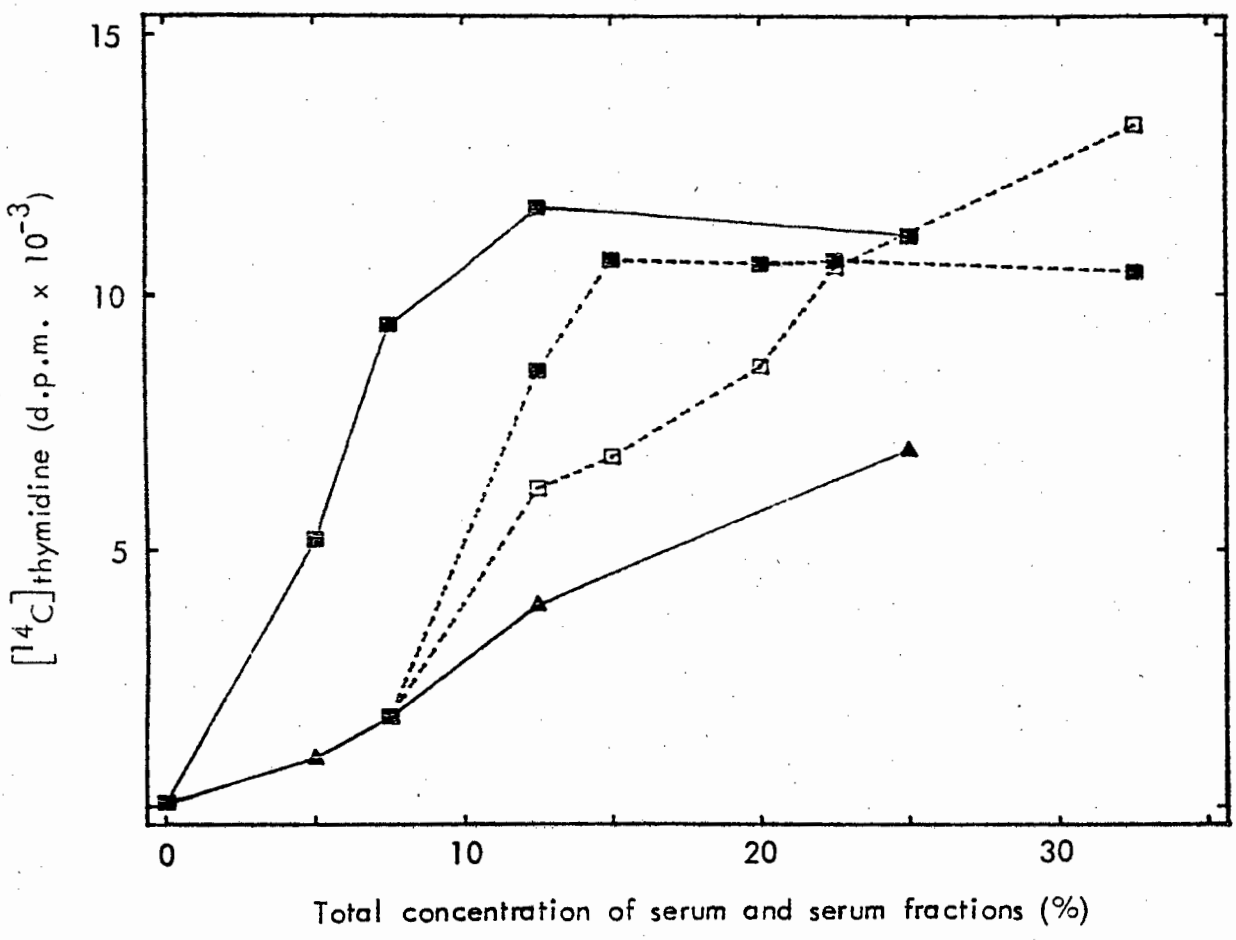


FIGURE 4.11 Reconstitution of kwashiorkor serum by an ultrafiltrate of normal serum.

Lexington, U.S.A.; MW cut-off < 500 daltons) and added to 5% kwashiorkor serum.

Dialysis of kwashiorkor serum against normal serum did not completely restore the ability of kwashiorkor serum to support PHA stimulation (Fig. 4.10), suggesting that a non-dialysable large molecular weight fraction was also deficient in kwashiorkor serum. Since kwashiorkor serum is invariably deficient in albumin, an experiment was performed in which purified human serum albumin (4g/100 ml) and an Amicon UM0 5 serum ultrafiltrate were added, singly or together, to 5% kwashiorkor serum. Fig. 4.12 shows that neither human serum albumin nor ultrafiltrate, on their own or combined, were able to support lymphocyte transformation in serum-free medium. The addition of albumin alone to 5% kwashiorkor serum did not enhance lymphocyte transformation responses, and the combination of albumin and ultrafiltrate did not significantly increase the values obtained with ultrafiltrate alone.

These experiments suggest that the reduced lymphocte transformation responses seen in cultures with kwashiorkor serum were mainly due to a deficiency of a low molecular weight serum fraction that was present in normal serum. It also seems probable that a large molecular weight substance was depleted in kwashiorkor serum; this did not appear to be serum albumin.

FURTHER CHARACTERISATION OF NORMAL SERUM FRACTIONS REQUIRED FOR MAXIMAL PHA LYMPHOCYTE TRANSFORMATION

With the knowledge that an ultrafiltrate of normal serum was able to supplement kwashiorkor serum to give near normal values, an attempt was made to define which fractions of normal serum were required for maximal lymphocyte transformation. A large molecular weight fraction of normal serum with a molecular weight of greater than 10 000 daltons was obtained

FIGURE 4.12

FIGURE 4.12 Lymphocyte blastogenic responses in media supplemented with sera or serum fractions alone and in combination.

This graph shows the PHA-induced $[^{14}C]$ -thymidine incorporation into lymphocytes incubated in media supplemented with sera or serum fractions alone or in combination. For comparative purposes human serum albumin concentrations are depicted in terms of "serum equivalents", 40 mg/ml of albumin being regarded as equivalent to 100% normal serum with respect to albumin concentration. The points depicted are the mean results of triplicate cultures and the symbols represent as follows:

AB serum alone (■——■);
kwashiorkor serum alone (▲——▲);
5% kwashiorkor serum and addition of UM05 ultrafiltrate of AB serum (△----△);
5% kwashiorkor serum and addition of UM05 ultrafiltrate and albumin (●----●);
5% kwashiorkor serum and addition of albumin (□----□);
albumin alone, ultrafiltrate alone or albumin and UM05 ultrafiltrate (●——●).

Note that (i) neither albumin, ultrafiltrate or the combination of the two in the absence of any serum is capable of supporting any lymphocyte transformation response;

(ii) the addition of albumin alone to 5% kwashiorkor serum results in no increase in lymphocyte transformation responses;

(iii) the addition of albumin to cultures supplemented with 5% kwashiorkor serum and normal serum ultrafiltrate does not augment lymphocyte transformation responses further.

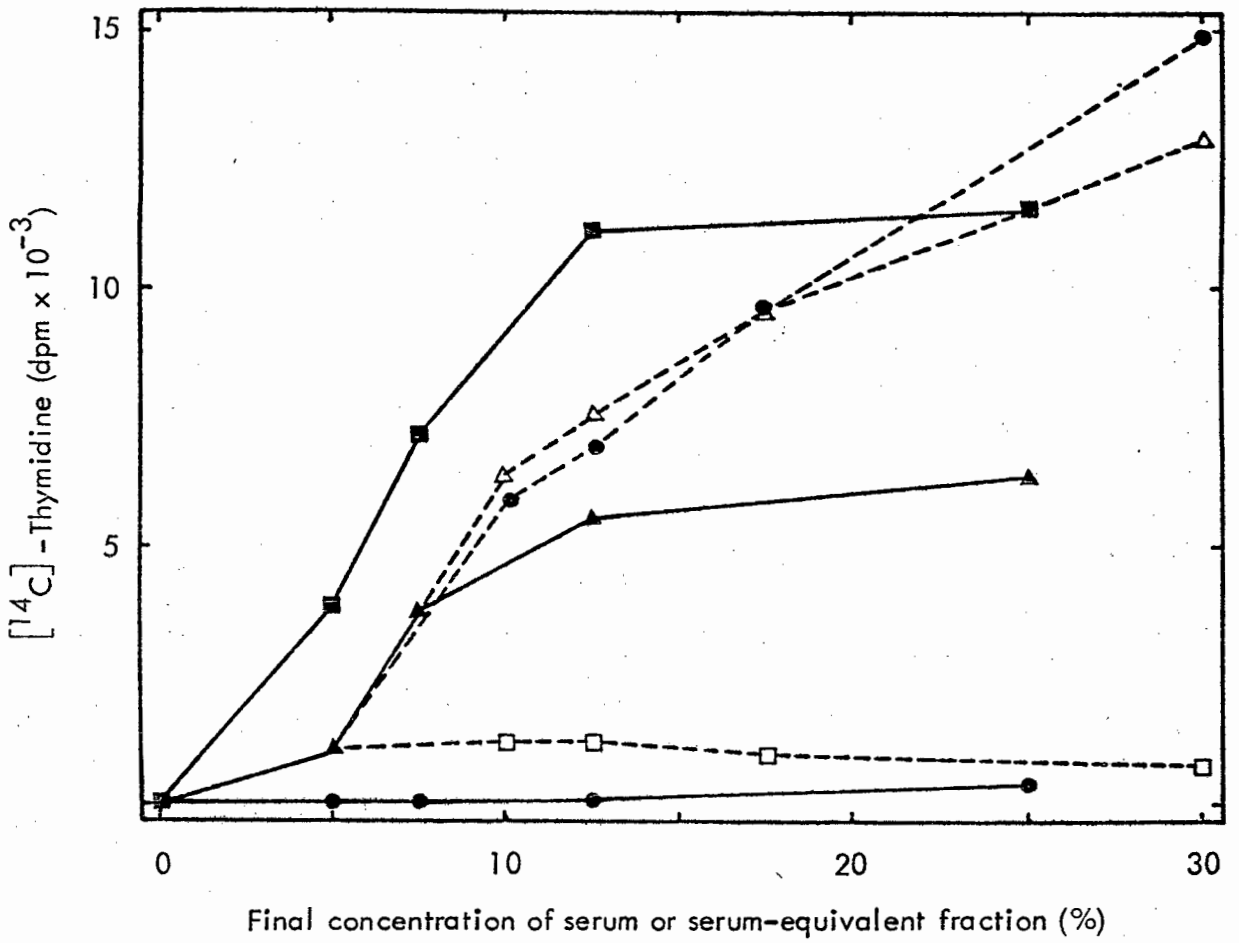


FIGURE 4.12 Lymphocyte blastogenic responses in media supplemented with sera or serum fractions alone and in combination.

by pressure dialysis using an Amicon PM 10 membrane. A portion of the dialysate of less than 10 000 daltons was then passed through an Amicon UMO 5 filter with a molecular weight of 500 daltons. The three fractions obtained, i.e. M.W. greater than 10 000 daltons (fraction A); M.W. 10 000 - 500 daltons (fraction B); and M.W. less than 500 daltons (fraction C) were all brought to their original volume and sterilised by filtration through a 0,45 µ millipore filter.

Lymphocyte cultures were established containing a 12,5% concentration of either the individual fractions, or a 12,5% concentration of each of the fractions in various combinations. The results obtained are summarised in Fig. 4.13. Lymphocyte transformation responses in medium supplemented with normal serum fractions on their own, or in incomplete combinations were lower than those observed in whole serum. The highest results were obtained when the high molecular weight Amicon fraction, on its own or in combination with low molecular weight fractions (A, A+B and A+C) was used to supplement media. The low molecular weight fractions on their own were very weak in supporting lymphocyte transformation (fractions B, C and B+C). The combination of the large molecular weight fraction of greater than 10 000 daltons and the small molecular weight fraction of less than 500 daltons (A + C) was able to support lymphocyte transformation as well as unfractionated serum.

Maximal *in vitro* lymphocyte responsiveness would thus appear to be dependent on serum supplementation, the essential component(s) of which are found both in the large molecular weight fraction (> 10 000 daltons) and in the low molecular weight range (< 500 daltons). From the preceding experiments it would appear that kwashiorkor serum is principally deficient in the low molecular weight component(s).

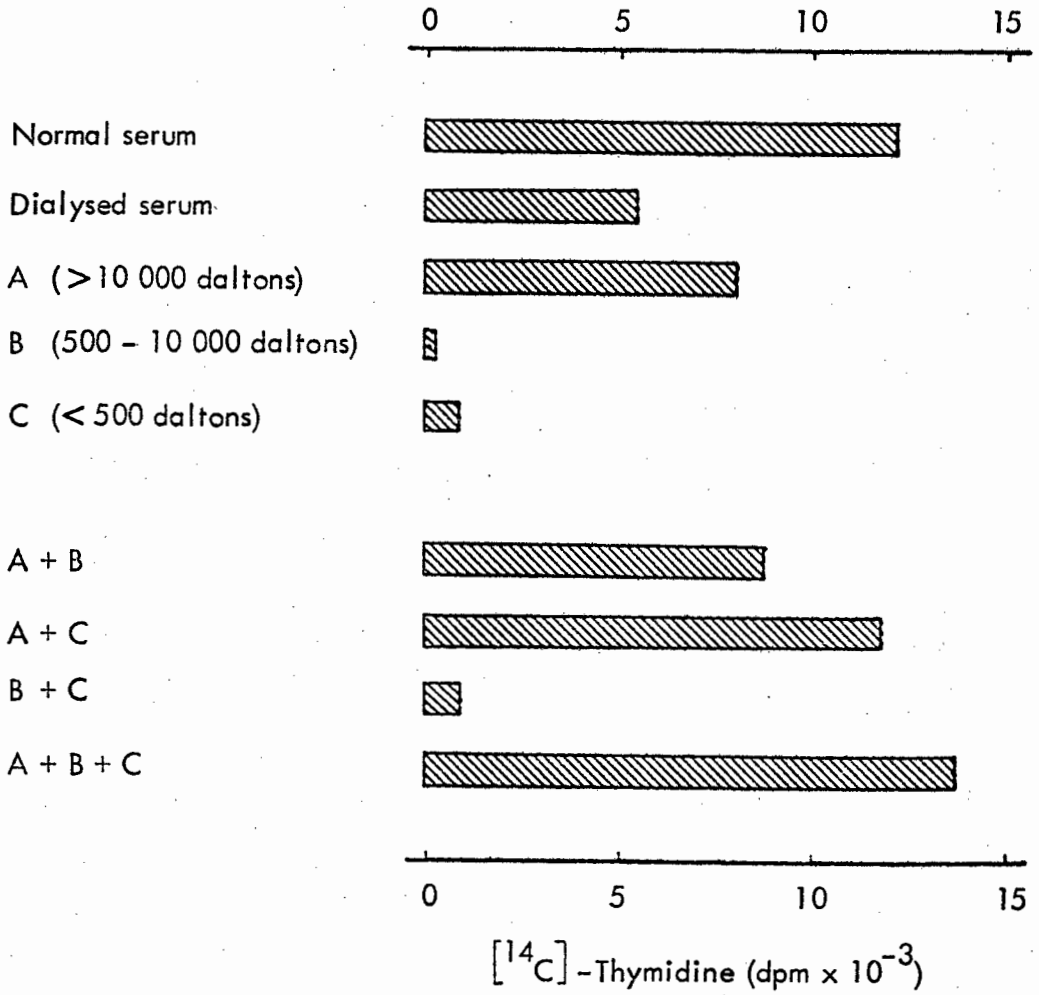


FIGURE 4.13 Lymphocyte transformation responses in normal serum and fractions of normal serum.

The bars in this graph represent the mean values of $[^{14}C]$ -thymidine incorporation, by triplicate cultures of 2×10^5 PHA-stimulated lymphocytes cultured in media supplemented with normal serum and fractions of normal serum as shown on the left. Serum fractions with the molecular weight cutoffs as shown were prepared with Amicon dialysis membranes. Note that lymphocyte transformation responses are more critically dependent on the large molecular weight components but that optimal responses are only obtained when both the lowest molecular weight fractions and the highest molecular weight fractions are combined.

THE EFFECTS OF KWASHIORKOR SERUM ON HUMAN SKIN FIBROBLAST GROWTH

Peripheral blood lymphocytes are generally regarded as cells in the resting phase of the cell cycle that remain in G1 until triggered to enter S phase by some mitogenic stimulus. Confluent, early-passage cultures of skin fibroblasts, on the other hand, are comprised of resting cells with an intrinsic proliferative potential that is activated by the simple procedure of passaging at subconfluent cell densities. Since lymphocytes and fibroblasts differ in their requirements for an exogenous mitogenic stimulus to pass from a state of dormancy to one of proliferation, it was of interest to ascertain whether or not they were equally susceptible to the inadequacies of kwashiorkor serum as a medium supplement.

Human preputial tissue was obtained at the time of circumcision, minced finely with scissors and plated as explants in 60 mm plastic dishes (Falcon, Oxnard, U.S.A.) under 5 ml of Dulbecco MEM (Gibco, New York, U.S.A.), supplemented with antibiotics, glutamine and 10% AB serum. When confluent, the fibroblast monolayers were trypsinised into a single cell suspension, washed with serum-free medium and replated at a density of 0,35 x 10^5 cells in 1,5 ml of medium into 35 mm dishes and supplemented with 10% AB serum or 10% kwashiorkor serum. Growth curves (Fig. 4.14) were constructed by trypsinising triplicate cultures after varying times of incubation at 37^oC in a humid atmosphere of 5% CO_2/95% air. The trypsinised cells were suspended in 10 ml of a particle-free, 3% cetrimide solution (Stewart and Goldstein, 1974) and the nuclei counted in an electronic particle counter (Coulter ZBl, settings: l/aperture= 1/4; l/amplification = 1; lower threshold = 12).

Cells cultured in AB serum grew exponentially between day 1 and day 4 with a doubling time of 0,74 days. Cells cultured in kwashiorkor serum showed a lag of approximately one day before exhibiting exponential growth

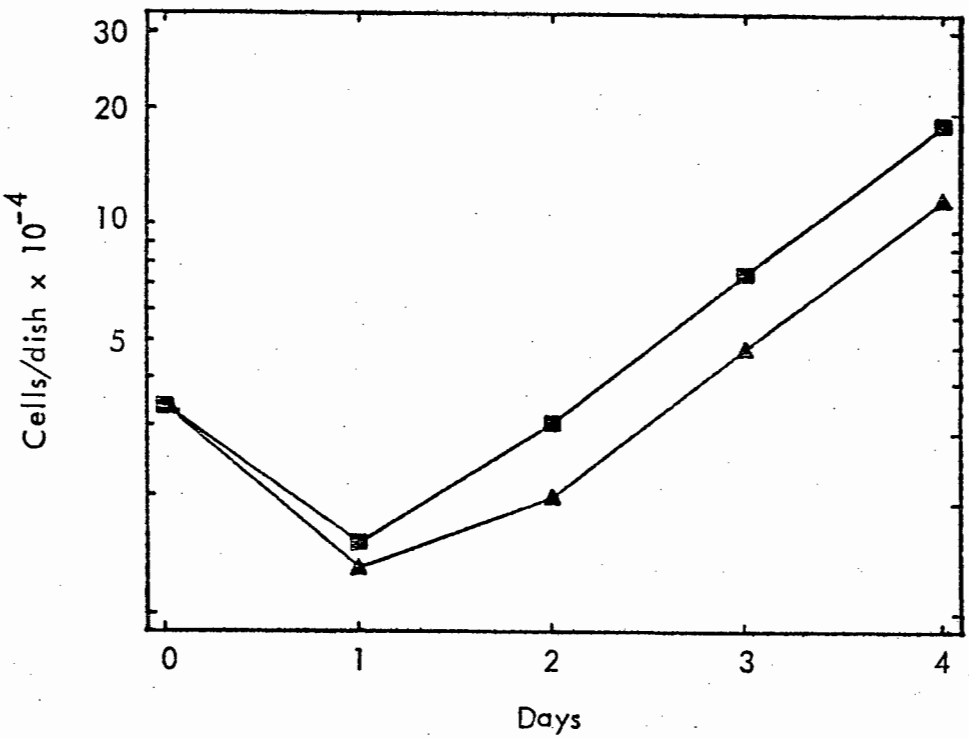


FIGURE 4.14 The effects of kwashiorkor serum on human skin fibroblast growth

The graph shows the mean values of duplicate cultures of human skin fibroblasts grown in medium supplemented with 10% AB serum (■——■) or 10% kwashiorkor serum (▲——▲) in which cell numbers were established at the times shown.
Note that fibroblast cultures in media supplemented with kwashiorkor serum show an initial growth lag between days 1 and 2, but thereafter exponential growth parallels that seen in cultures supplemented with AB serum.

at the same rate. It is, therefore, concluded that supplementation of the culture medium with kwashiorkor serum placed fibroblasts at an initial disadvantage which they were able to overcome by "conditioning" their own medium.

ATTEMPTS TO IDENTIFY THE FACTOR(S) IN THE ULTRAFILTRATE OF NORMAL SERUM REQUIRED FOR OPTIMAL LYMPHOCYTE RESPONSIVENESS TO PHA

The observations in the preceding sections showed that lymphocte responses to mitogens required, in addition to tissue culture medium, both a large and a small molecular weight component of normal serum. Kwashiorkor serum is inadequate in its ability to support lymphocyte transformation because of a deficiency in the low molecular weight component(s), and an ultrafiltrate of normal serum with a molecular weight of less than 500 daltons was able to reconstitute kwashiorkor serum. Attempts were therefore made to identify this factor or these factors in the ultrafiltrate and to characterise its (their) properties as far as possible.

As kwashiorkor serum was available in only limited quantities, the addition of treated ultrafiltrate was compared with untreated ultrafiltrate and used to reconstitute dialysed AB serum. The previous experiments had shown that dialysed AB serum behaved in a similar fashion to kwashiorkor serum in that submaximal blastogenic responsiveness was obtained when it was used as a serum supplement in PHA-stimulated lymphocyte cultures.

Physical Properties

Ultrafiltrate of normal AB serum was treated in the following ways:-

The pH of the ultrafiltrate was lowered to 4 and 2 by the addition of 0,1N formic acid and maintained at 4^{o}C for 1 hour. The sample was

TABLE 4.2

Additions to Lymphocyte Cultures	$[^{14}C]$ -Thymidine Incorporation (% of maximum)
Dialysed AB serum (7,5%)	4,8
+ 15% AB uf* (untreated)	100 (max)
+ 15% AB uf (pH 4 for 1 hr)	2,1
+ 15% AB uf (pH 2 for 1 hr)	0,5
+ 15% AB uf (pH 10 for 1 hr)	91,8
+ 15% AB uf (H_2O_2 + lactoperoxidase)	82,5
+ 15% AB uf (H_2O_2 + lactoperoxidase + pH 2 for 1 hr)	10,3
+ 15% AB uf (direct sunlight for 1 hr)	100
+ 15% AB uf ($100^{o}C$ for 1 hr)	100
+ 15% AB uf ($500^{o}C$ for 5 hr)	0,7
+ 15% AB uf (lyophilised untreated)	85,5

* uf = ultrafiltrate

then readjusted to a pH of 7,5 by the addition of ammonia solution and the preparation lyophilised and redissolved in distilled water to its original volume.

Alkali treatment of the ultrafiltrate was accomplished by the addition of 1N NaOH at pH 10, with readjustment, after 1 hour at 4°C, to pH 7,5 with 1N HCl.

Ultrafiltrate was exposed to direct sunlight for 1 hour.

Oxidation of the ultrafiltrate was achieved by the addition of H_2O_2 and lactoperoxidase linked to Sepharose 4B. A portion of the oxidised sample was acid-treated for 1 hour as described above.

Heat-stability was assessed by heating a sample of ultrafiltrate for 1 hour at 100°C.

A sample of ultrafiltrate was completely ashed by heating to 500°C for 5 hrs and then reconstituted in distilled water.

The results of normal lymphocyte responsiveness to PHA cultures containing 7,5% dialysed AB serum, to which 15% ultrafiltrate treated as above and untreated ultrafiltrate were added, are summarised in Table 4.2.

The ultrafiltrate was unaffected by alkali treatment, was heat-stable and was not affected by lyophilisation, exposure to light or oxidation. Acid treatment abolished its ability to reconstitute dialysed serum. Ashing of the sample at 500°C for 5 hrs removed the active factor(s) in the ultrafiltrate.

Fractionation and Extraction

Attempts to isolate the active principle were made by passing the ultrafiltrate through various affinity columns and by solvent extraction. The ultrafiltrate was added to a DEAE 52 column equilibrated with 0,005 M NH_4HCO_3 buffer at pH 7,9 and a stepwise elution with 0,05 M - 0,2 M NH_4HCO_3 performed.

Fractions were lyophilised and brought to their original volume and pH before testing. Results showed that none of the active factors were removed by passage through the column.

Samples of ultrafiltrate were also applied to Ag 50W and Biogel P6 columns. Because of the acid buffers used in these procedures, activity was lost in all fractions.

p-chloromercurobenzoate (PCMB) was linked to cyanogen bromide-activated Sepharose 4B and equilibrated with phosphate-buffered saline (pH 7,4). Ultrafiltrate was passed through a column packed with the PCMB - Sepharose using phosphate-buffered saline as a running buffer. None of the ultrafiltrate's activity was removed by passage through this column.

Activated charcoal saturated with adenine was washed with ethanol : ammonia : water (45 : 5 : 50) and then distilled water until the pH was neutral. Three millilitres of ultrafiltrate were mixed with 0,1 ml of charcoal for 1 hour at room temperature and separated by centrifugation. Ultrafiltrate which had been treated in this way lost 60% of its ability to reconstitute dialysed AB serum. This experiment indicated that some of the active principle in the ultrafiltrate may have been bound by the activated charcoal.

Solvent extraction was performed by lyophilising the ultrafiltrate and suspending the freeze-dried material in various solvents. The insoluble material was removed by centrifugation and the extracted portion by evaporation of the solvent under a stream of nitrogen. The insoluble material was reconstituted with distilled water and the extracted substance in T MEM. These fractions were then added at a concentration of 25% to PHA-stimulated lymphocyte cultures supplemented with 7,5% dialysed AB serum. The $[^{14}C]$ -thymidine incorporated in the cultures supplemented with the soluble and insoluble fractions was expressed as a percentage of

TABLE 4.3

Additions to Lymphocyte Cultures		$[^{14}C]$ -Thymidine Incorporation (% of maximum)
Dialysed AB serum (7,5%)		17
+ 25% AB uf*	(untreated)	100 (max)
+ 25% AB uf	(acetone extracted)	67
+ 25% AB uf	(acetone insoluble)	22
+ 25% AB uf	(ethanol extracted)	0
+ 25% AB uf	(ethanol insoluble)	58
+ 25% AB uf	(methanol extracted)	82
+ 25% AB uf	(methanol insoluble)	16
+ 25% AB uf	(ether extracted)	37
+ 25% AB uf	(ether insoluble)	54

* uf = ultrafiltrate

maximal enhancement of lymphocyte responsiveness achieved by the addition of untreated freeze-dried ultrafiltrate. The results are shown in Table 4.3. Examination of this table shows that methanol and, to a lesser extent, acetone are reasonably effective in extracting the active factors.

Addition of Various Supplements to Kwashiorkor Serum

In addition to the usual amino acids, trace elements and growth substances present in tissue culture medium, various other elements and substances were added to cultures containing kwashiorkor serum to see whether any enhancement of lymphocyte responsiveness could be achieved. These are listed below at the concentration at which they were added:-

1,8	mMol/l	$CaCl_2$
0,8	mMol/l	$ZnCl_2$
7,0	µMol/l	$ZnSO_4.7H_2O$
0,4	mMol/l	$FeCl_2$
3,6	µMol/l	$FeSO_4.7H_2O$
2,8	mMol/l	Ca lactate
0,99	mMol/l	pyruvic acid.

None of these substances had any effect in enhancing kwashiorkor serum's ability to support lymphocyte blastogenesis.

Similarly, the substances listed below were added to dialysed AB serum to determine whether they may constitute in part the active principle(s) present in the ultrafiltrate of normal serum which was lost during dialysis.

1,8	mMol/l	$CaCl_2$
0,8	mMol/l	$ZnCl_2$
0,4	mMol/l	$FeCl_2$
20	µMol/l	$MnSO_4$
3,6	µMol/l	Biotin
2,3	µMol/l	B_{12}
31,0	µMol/l	PABA
31,2	µMol/l	Choline
9,9	µMol/l	Folic acid
42,5	µMol/l	Inositol
35,8	µMol/l	Nicotinamide
9,2	µMol/l	Pantothenic acid
21,5	µMol/l	Pyridoxine
1,2	µMol/l	Riboflavin
13,0	µMol/l	Thiamine
2,5	mMol/l	Deoxyguanosine
2,5	mMol/l	Deoxyadenosine

None of these substances were able to replace the ultrafiltrate of normal serum in reconstituting dialysed AB serum.

Although these experiments were not conducted with kwashiorkor serum but with dialysed AB serum, it seems reasonable to assume that for this purpose these are comparable, as the ultrafiltrate of normal serum is capable of enhancing the serum supportive capacity of both to a comparable degree.

The following conclusions can thus be drawn from these experiments. The ultrafiltrate of normal serum contains a factor or factors which is (are) required for normal maximal lymphocyte responsiveness to PHA in cultures. This factor is acid-labile and can be destroyed by ashing at

500°C. It is heat-stable and is resistant to lyophilisation, oxidation, sunlight and alkali treatment. It can be partially extracted by activated charcoal and methanol. It does not appear to be one of the substances ordinarily present in tissue culture medium or those substances which were added to cultures and are listed above.

DISCUSSION

The experimental results reported in this chapter confirm and extend the previous observations in Chapter 3. Serum samples from children with kwashiorkor were consistently deficient in their ability to support lymphocyte transformation *in vitro* when compared to a reference pool of normal adult AB serum.

The kwashiorkor serum effect was not mitogen-specific inasmuch as it affected blastogenic responses to PHA, PWM, Con A (Fig. 4.1) and allogeneic cells (Fig. 3.6). Lectin dose-response experiments showed that the serum did not act by inactivating or otherwise reducing the effective concentration of the mitogen (Fig. 4.1).

Kwashiorkor serum supplements gave inadequate blastogenic responses, not only as measured by $[^{14}C]$ -thymidine incorporation into whole cells, but also as determined by nuclear-sizing techniques (Fig. 4.5) and histological evaluation of autoradiographs (Fig. 4.7). It is concluded, therefore, that the serum effect was exerted directly upon nuclear DNA synthesis and not upon transport of thymidine into stimulated cells nor upon the size of intracellular thymidine pools.

Prolonged storage and repeated freezing and thawing did not improve the supportive quality of kwashiorkor serum, suggesting that the effect of kwashiorkor serum was not due to the presence of a labile inhibitor of blastogenesis. More definitive mixing experiments (Fig. 4.9) showed that the defect in kwashiorkor serum was due to a deficiency of some

factor(s) essential for optimal lymphocyte transformation and not due to the presence of an inhibitor. This observation is intuitively consistent with the concept of kwashiorkor as a deficiency disease in which nutritional deprivation hampers cell growth and the cellular proliferation that this implies (Ramalingaswami, 1969).

Humoral factors affecting lymphocyte transformation have been extensively reviewed (Nelson and Gatti, 1976). These factors have been recognised in a wide variety of pathological conditions, in normal human serum and in animal serum. In only a few instances have the serum factors affecting lymphocyte transformation been fully characterised and little distinction has been made between true inhibitors and the absence of essential factors. The characterised inhibitory factors fall into three main groups:-

1) α globulins either naturally occurring or in pathological conditions;
2) immunoglobulins which may have specificity for a particular antigen;
3) low molecular weight factors including drugs, hormones and possibly toxic metabolites.

The results of the experiments in which kwashiorkor serum was added to normal serum indicate that the reduced lymphocyte transformation seen in kwashiorkor is not due (in the majority of cases) to an inhibitor in the serum and therefore does not come under consideration in these groups.

The results of viable cell counts (Fig. 4.3), the reversibility of the kwashiorkor serum effect (Fig. 4.4) and the results of serum mixing experiments (Fig. 4.9) exclude the trivial explanation that kwashiorkor serum was simply cytotoxic for lymphocytes. The precise manner in which the deficiency in kwashiorkor serum acted, however, is obscure. The serum switch experiments (Fig. 4.8) indicated that optimal transformation required the presence of normal serum throughout both the initial and late phases of the mitogenic response, although there was some indication

that the initial, pre-synthetic phase of DNA assembly was preferentially affected by the deficiency in kwashiorkor serum. It is possible that better synchronised cultures might have identified, with more certainty, a specified period in the cell cycle during which stimulated cells were particularly vulnerable to the effects of the deficiency.

When PHA-stimulated lymphocytes were incubated in medium supplemented with kwashiorkor serum and harvested after 2 hr pulses of [^{3}H]-thymidine or [^{3}H]-uridine at different times after initiation of culture, cellular incorporation of both radioactive nucleosides was depressed compared to cells incubated with normal serum (Fig. 4.2). These results indicate that the synthesis of both RNA and DNA were affected by the inadequacy of the kwashiorkor serum. Since RNA synthesis precedes DNA synthesis in this system and is known to be required for S phase DNA synthesis (Mueller, 1969), it is possible that the kwashiorkor serum effect on DNA synthesis was secondary to its effect upon prerequisite synthesis of RNA or other macromolecules.

The absolute requirement for serum or some small serum substance for growth of mammalian cells was recognised many years ago (Eagle, 1965). Ling, Spicer, James and Williamson (1965) established that dialysed serum did not support blastic transformation of human lymphocytes. The experiments shown in Fig. 4.13 indicate firstly that dialysis of normal serum abolishes the ability to support maximal PHA-induced lymphocyte transformation and that the absence of low molecular weight substances of less than 500 daltons is critical in this respect.

The precise chemical nature of the compound(s) that is (are) lacking in kwashiorkor serum has not as yet been defined. This could, to a large extent, be supplied by dialysis of deficient serum against AB serum (Fig. 4.10) and by the addition of an ultrafiltrate of AB serum (Fig. 4.11), indicating that the kwashiorkor serum was mainly deficient in

some low molecular weight (< 500 daltons) component(s) required for optimal transformation. The fact that these procedures failed, in certain cases, to restore deficient sera to complete normality indicated that large molecular weight compound(s) might also have been involved.

Since hypoalbuminaemia is a cardinal manifestation of kwashiorkor (Whitehead, Coward and Lunn, 1973) and since albumin has been used successfully as a serum substitute for lymphocte transformation (Spieker-Polet and Polet, 1976), it was reasonable to suspect that a deficiency of this protein might have been partly responsible for the serum defect. This, however, did not prove to be the case, since the addition of albumin did not improve the supportive quality of kwashiorkor serum (Fig. 4.12).

Furthermore, it has been postulated that an increased free serum cortisol in kwashiorkor serum due to low albumin levels and therefore decreased albumin-bound cortisol may be a factor in the immunosuppression of kwashiorkor (Schonland, Shanley, Loening, Parent and Coovadia, 1972). The results presented here do not support this hypothesis on two counts, firstly that kwashiorkor serum is not inhibitory and secondly that the addition of albumin to kwashiorkor serum did not enhance responses.

In Table 4.1 it is evident that lymphocytes transform maximally in RPMI 1640 medium containing bicarbonate and Hepes buffers. Statistical analysis shows no interaction between kwashiorkor serum and either the medium or the buffers. Inspection of this table shows that maximal differences between kwashiorkor and AB serum are seen in Tris-buffered Eagle's medium and this medium was used because of its greater discrimination. Direct pH determinations of cultures containing kwashiorkor and normal serum in T MEM show no differences at any time period during incubation suggesting that kwashiorkor serum is not inhibitory because

of a decreased buffering capacity.

Since the kwashiorkor serum deficiency was manifest in the presence of various tissue culture media (Table 4.1), the low molecular weight component(s) that were lacking were not among those amino acids, vitamins, salts, energy substrates or other known growth factors normally included in the formulation of such media. The fact that tissue culture media lack low molecular weight materials that are necessary for optimum blastogenesis and are supplied by normal serum has been borne out by observations that AB serum that had been dialysed against RPMI 1640, Eagle's MEM or medium 199 lost its ability to support lymphocyte transformation.

Skin fibroblasts, although initially retarded in their *in vitro* proliferation in the presence of kwashiorkor serum, were able to make good the deficiency by "conditioning" their medium (Fig. 4.14). Lymphocytes, it would seem, lack this capacity.

The biochemical characteristics of this deficient substance have not been defined apart from the establishment that it is less than 500 daltons in molecular weight.

Experiments conducted with the ultrafiltrate of normal serum have not been helpful in this regard, other than to establish that the active property (ies) is (are) acid-labile, and can be destroyed by heating to 500^{o}C. Preliminary extraction procedures suggest that methanol-extraction may be the most promising means of concentrating and identifying this low molecular weight substance.

Pickert and Thaler (1973) have shown that a peptide of approximately 300 daltons M.W. (glyc-hist-lys) extracted from normal serum stimulates growth of neoplastic cells. Thymic factors present in normal serum have been characterised by Bach and Carnaud (1975). Thymic hormone has a molecular weight of approximately 1 000 daltons and it is attractive to

speculate, knowing the severe degree of thymic atrophy in kwashiorkor, that decreased levels of circulating thymic hormone are responsible for the serum effect in kwashiorkor.

Although serum factors that support lymphocyte transformation are complex and their positive and complete identification represents a formidable task, it is to be hoped that this final characterisation will be achieved. The availability of such compounds in a pure, biologically active form would contribute much to the understanding and the management of the immunodeficiency of kwashiorkor.

APPENDIX

A. 1 KWASHIORKOR PATIENTS

CASE No. 1

Diagnosis:	Marasmic Kwashiorkor
Sex:.	M
Age (months):	18
Weight (kg):	6,000
Expected weight for age (%):	53
Oedema:	++
Dermatitis:	++
Hair changes:	+
Hepatomegaly:	3 cm
McLaren score:	11
Cultures: stool:	Candida albicans
Result:	Recovered

Laboratory Results:

		Admission	2 weeks	4 weeks	6 weeks
Total protein	(g/100 ml)	4,87	6,84	7,22	7,55
Albumin	(g/100 ml)	2,24	3,28	3,91	4,08
α_1 globulin	(g/100 ml)	0,2	0,32	0,22	0,21
α_2 globulin	(g/100 ml)	0,4	0,76	0,63	0,64
β globulin	(g/100 ml)	0,51	1,12	1,04	1,11
γ globulin	(g/100 ml)	1,52	1,37	1,41	1,50
Hb	(g/100 ml)	9,6			
Total W.C.C.	(mm^3)	12 400			
PMN	(%)	82			

		Admission	2 weeks	4 weeks	6 weeks
Lymphocytes	(%)	14			
Monocytes	(%)	1			
Eosinophils	(%)	3			
Total lymph. count	(mm^3)	1736			
NBT (unstimulated)	(%)	21			
(stimulated)	(%)	40			
Blood group		AB +ve			
IgG	(mg/100 ml)	2180	1630	1200	1240
IgM	(mg/100 ml)	268	320	270	208
IgA	(mg/100 ml)	258	306	220	204
IgE	(u/ml)	1087	1129		538
C3	(mg/100 ml)	nil	188	164	149

Lymphocyte Transformation (dpm $[^{14}C]$ -thymidine)

Lymph	Serum				
Control	AB	36571	40245	29378	16068
Control	Patient	10233	28406	22890	18165
Patient	AB	48194	39891	39931	55812
Patient	Patient	11453	26738	17423	46729

Mixed Lymphocyte Cultures (dpm $[^{14}C]$ -thymidine)

A = Patient B = Control

Lymph	Serum			
ABm	AB	2348	1262	17611
AmB	AB	1753	2549	5305
AmBm	AB	19	14	33
AB	AB	2860	4567	18570
ABm	Patient	225	293	13729
AmB	Patient	-	895	4930
AmBm	Patient	14	-	31
AB	Patient	-	-	14453

CASE No. 2

Diagnosis:	Kwashiorkor and Lactose Intolerance
Sex:	F
Age (months):	18
Weight (kg):	6,740
Expected weight for age (%):	61,2
Oedema:	+
Dermatitis:	++++
Hair changes:	+
Hepatomegaly:	2 cm
McLaren score:	12
Cultures: stool:	Ascaris lumbricoides
Result:	Recovered

Laboratory Results:

		Admission	6 weeks
Total protein	(g/100 ml)	5,14	7,75
Albumin	(g/100 ml)	1,97	3,45
α_1 globulin	(g/100 ml)	0,27	0,28
α_2 globulin	(g/100 ml)	0,64	1,10
β globulin	(g/100 ml)	0,68	0,93
γ globulin	(g/100 ml)	1,57	2,00
Hb	(g/100 ml)	9,0	
Total W.C.C.	(mm^3)	7 100	
PMN	(%)	49	
Lymphocytes	(%)	48	
Monocytes	(%)	3	
Total lymph. count	(mm^3)	3408	

		Admission	6 weeks
IgG	(mg/100 ml)	1161	2062
IgM	(mg/100 ml)	380	297
IgA	(mg/100 ml)	205	76
IgE	(u/ml)	1405	62
C3	(mg/100 ml)	179	176

PHA Lymphocyte Transformation (dpm $[^{14}C]$ -thymidine)

Lymph	Serum		
Control	AB	23052	31183
Control	Patient	5629	15131
Patient	AB	30082	28068
Patient	Patient	9187	12505

CASE No. 3

Diagnosis:	Kwashiorkor and Pneumonia
Sex:	M
Age (months):	20
Weight (kg):	9,400
Expected weight for age (%):	80
Oedema:	++++
Dermatitis:	++
Hair changes:	-
Hepatomegaly:	2 cm
McLaren score:	10
Cultures: stool:	Giardia lamblia
Chest X-ray:	Right upper lobe pneumonia
Result:	Recovered

Laboratory Results:

		Admission	2 weeks	4 weeks
Total protein	(g/100 ml)	4,81	7,2	8,09
Albumin	(g/100 ml)	2,15	3,57	3,97
α_1 globulin	(g/100 ml)	0,24	0,28	0,28
α_2 globulin	(g/100 ml)	0,56	1,1	1,09
β globulin	(g/100 ml)	0,71	1,07	1,25
γ globulin	(g/100 ml)	1,15	1,18	1,5
Hb	(g/100 ml)	10,2	9,3	
Total W.C.C.	(mm^3)	10 700	8 400	
PMN	(%)	77	34	
Lymphocytes	(%)	22	65	
Monocytes	(%)	2	1	

		Admission	2 weeks	4 weeks
Total lymph. count	(mm^3)	2354	5460	
NBT (unstimulated)	(%)	70		
Blood group		0 +ve		
Anti-A agglutinins (titre)		1 : 512		
Anti-B agglutinins (titre)		1 : 256		
IgG	(mg/100 ml)	1210	1150	1190
IgM	(mg/100 ml)	261	264	336
IgA	(mg/100 ml)	249	286	216
IgE	(u/ml)	36		
C3	(mg/100 ml)	71	160	146

PHA Lymphocyte Transformation (dpm $[^{14}C]$ -thymidine)

Lymph	Serum			
Control	AB	24156	38013	24177
Control	Patient	7631	21381	5731
Patient	AB	23131	24164	32602
Patient	Patient	3865	6558	2682

Mixed Lymphocyte Cultures (dpm $[^{14}C]$ -thymidine)

A = Patient B = Control

Lymph	Serum			
ABm	AB	14284	12081	4824
AmB	AB	14297	13060	4590
AmBm	AB	35	24	5
AB	AB	10296	18361	6649
ABm	Patient	2643		6233
AmB	Patient	3166		1988
AmBm	Patient	-		2
AB	Patient	3865		4952

CASE No. 4

Diagnosis:	Kwashiorkor
Sex:	F
Age (months):	21
Weight (kg):	7,880
Expected weight for age (%):	67,6
Oedema:	++++
Dermatitis:	+
Hair changes:	-
Hepatomegaly:	2 cm
McLaren score:	11
Cultures: stool:	Salmonella
Result:	Recovered

Laboratory Results:

		Admission
Total protein	(g/100 ml)	5,49
Albumin	(g/100 ml)	1,79
α_1 globulin	(g/100 ml)	0,28
α_2 globulin	(g/100 ml)	0,92
β globulin	(g/100 ml)	0,85
γ globulin	(g/100 ml)	1,63
Hb	(g/100 ml)	9,8
Total W.C.C.	(mm^3)	11 400
PMN	(%)	33
Lymphocytes	(%)	61
Monocytes	(%)	6
Total lymph. count	(mm^3)	6710

		Admission
NBT (unstimulated)	(%)	1
(stimulated)	(%)	40
Blood group		0 +ve
Anti-A agglutinins (titre)		1 : 128
Anti-B agglutinins (titre)		1 : 16
IgG	(mg/100 ml)	2220
IgM	(mg/100 ml)	207
IgA	(mg/100 ml)	176
IgE	(u/ml)	50
C3	(mg/100 ml)	161

PHA Lymphocyte Transformation (dpm $[^{14}C]$ -thymidine)

Lymph	Serum	
Control	AB	38843
Control	Patient	23722
Patient	AB	41870
Patient	Patient	13826

Mixed Lymphocyte Cultures (dpm $[^{14}C]$ -thymidine)

A = Patient B = Control

Lymph	Serum	
ABm	AB	9381
AmB	AB	8965
AmBm	AB	99
AB	AB	18631
ABm	Patient	5240
AmB	Patient	3489
AmBm	Patient	26
AB	Patient	6663

CASE No. 5

Diagnosis:		Kwashiorkor and Pneumonia
Sex:		F
Age (months):		22
Weight (kg):		9,110
Expected weight for age (%):		77,2
Oedema:		++++
Dermatitis:		++
Hair changes:		+
Hepatomegaly:		1 cm
McLaren score:		11
Cultures:	skin:	E. coli and Staphylococcus albus
Chest X-ray:		Right middle lobe pneumonia
Result:		Recovered

Laboratory Results:

		Admission	2 weeks	4 weeks
Total protein	(g/100 ml)	5,7	8,15	7,48
Albumin	(g/100 ml)	2,31	3,73	3,72
α_1 globulin	(g/100 ml)	0,28	0,26	0,26
α_2 globulin	(g/100 ml)	0,73	1,08	0,81
β globulin	(g/100 ml)	0,78	1,18	1,04
γ globulin	(g/100 ml)	1,6	1,91	1,65
Hb	(g/100 ml)	10,3	10,5	12,2
Total W.C.C.	(mm^3)	9 100	9 800	10 200
PMN	(%)	21	32	
Lymphocytes	(%)	74	64	
Monocytes	(%)	5	2	

		Admission	2 weeks	4 weeks
Eosinophils	(%)		2	
Total lymph. count	(mm^3)	6734	6272	
NBT (unstimulated)	(%)	24		
Blood group		A +ve		
Anti-B agglutinins (titre)		1 : 128		
IgG	(mg/100 ml)	1690	1690	1700
IgM	(mg/100 ml)	199	246	242
IgA	(mg/100 ml)	240	252	175
IgE	(u/ml)	43		
C3	(mg/100 ml)	133		215

PHA Lymphocyte Transformation (dpm $[^{14}C]$ -thymidine)

Lymph	Serum			
Control	AB	38509	23660	48028
Control	Patient	15172	22842	36971
Patient	AB	16227	14701	40378
Patient	Patient	4088	12147	31773

Mixed Lymphocyte Cultures (dpm $[^{14}C]$ -thymidine)

A = Patient B = Control

Lymph	Serum			
ABm	AB	7032	8578	3105
AmB	AB	8195	3197	4926
AmBm	AB	60	20	60
AB	AB	12599	12371	-
ABm	Patient	475	4484	8026
AmB	Patient	845	1445	5065
AmBm	Patient	52	-	-
AB	Patient	1626	7468	-

		Admission	2 weeks	6 weeks
IgG	(mg/100 ml)	860	1280	1320
IgM	(mg/100 ml)	172	95	146
IgA	(mg/100 ml)	103	153	52
IgE	(u/ml)	26		36
C3	(mg/100 ml)	52	210	166

PHA Lymphocyte Transformation (dpm $[^{14}C]$ -thymidine)

Lymph	Serum			
Control	AB	22612	53788	27596
Control	Patient	11835	51651	29572
Patient	AB	11417	48274	37262
Patient	Patient	4259	43767	32528

CASE No. 7

Diagnosis:		Kwashiorkor
Sex:		F
Age (months):		23
Weight (kg):		9,010
Expected weight for age (%):		74,5
Oedema:		++++
Dermatitis:		++++
Hair changes:		+
Hepatomegaly:		3 cm
McLaren score:		13
Cultures:	throat:	Candida albicans
	stool:	Trichomonas
Result:		Recovered

Laboratory Results:

		Admission	2 weeks	6 weeks
Total protein	(g/100 ml)	4,02	6,84	8,44
Albumin	(g/100 ml)	1,37	2,92	3,75
α_1 globulin	(g/100 ml)	0,23	0,30	0,28
α_2 globulin	(g/100 ml)	0,56	0,76	1,17
β globulin	(g/100 ml)	0,53	1,14	0,84
γ globulin	(g/100 ml)	1,33	1,72	2,41
Hb	(g/100 ml)	10,7		
Total W.C.C.	(mm^3)	7 700		
PMN	(%)	83		
Lymphocytes	(%)	15		
Monocytes	(%)	2		
Total lymph. count	(mm^3)	1155		

		Admission	2 weeks	6 weeks
IgG	(mg/100 ml)	1740	1811	2483
IgM	(mg/100 ml)	122	171	157
IgA	(mg/100 ml)	135	133	105
IgE	(u/ml)	192	309	177
C3	(mg/100 ml)	132	197	202

PHA Lymphocyte Transformation (dpm $[^{14}C]$ -thymidine)

Lymph	Serum			
Control	AB	30206	39238	33833
Control	Patient	11898	7821	32642
Patient	AB	20411	32373	34502
Patient	Patient	17498	18670	35407

CASE No. 8

Diagnosis:	Kwashiorkor
Sex:	M
Age (months):	24
Weight (kg):	10,030
Expected weight for age (%):	79
Oedema:	++++
Dermatitis:	+
Hair changes:	-
Hepatomegaly:	2 cm
McLaren score:	11
Cultures: stool:	Trichuris trichuria
Result:	Recovered

Laboratory Results:

		Admission	2 weeks	4 weeks
Total protein	(g/100 ml)	4,67	7,10	6,95
Albumin	(g/100 ml)	1,81	3,24	3,53
α_1 globulin	(g/100 ml)	0,24	0,32	0,25
α_2 globulin	(g/100 ml)	0,87	1,23	0,85
β globulin	(g/100 ml)	0,57	1,11	0,92
γ globulin	(g/100 ml)	1,18	1,20	1,41
Hb	(g/100 ml)	10,3		
Total W.C.C.	(mm^3)	5 200		
PMN	(%)	42		
Lymphocytes	(%)	55		
Eosinophils	(%)	3		
Total lymph. count	(mm^3)	2860		

		Admission	2 weeks	4 weeks
NBT (unstimulated)	(%)	36		
IgG	(mg/100 ml)	1260	1890	1940
IgM	(mg/100 ml)	245	252	172
IgA	(mg/100 ml)	127	105	22
IgE	(u/ml)	404		719
C3	(mg/100 ml)	137	217	119

PHA Lymphocyte Transformation (dpm $[^{14}C]$ -thymidine)

Lymph	Serum			
Control	AB	52080	38133	40245
Control	Patient	23019	27344	42446
Patient	AB	53977	41511	27738
Patient	Patient	12479	24330	15732

Mixed Lymphocyte Cultures (dpm $[^{14}C]$ -thymidine)

A = Patient B = Control

Lymph	Serum			
ABm	AB	9498	6135	8665
AmB	AB	4737	8688	9645
AmBm	AB	10	93	20
AB	AB	7683	16357	17066
ABm	Patient	2535	9009	6300
AmB	Patient	592	7818	4956
AmBm	Patient	-	-	-
AB	Patient	1692	-	-

CASE No. 9

Diagnosis:	Kwashiorkor
Sex:	F
Age (months):	30
Weight (kg):	9,220
Expected weight for age (%):	68,6
Oedema:	++++
Dermatitis:	+++
Hair changes:	+
Hepatomegaly:	3 cm
McLaren score:	12
Result:	Recovered

Laboratory Results:

		Admission	2 weeks	6 weeks
Total protein	(g/100 ml)	5,07	7,84	7,05
Albumin	(g/100 ml)	1,86	3,7	3,79
α_1 globulin	(g/100 ml)	0,33	0,32	0,2
α_2 globulin	(g/100 ml)	0,82	1,03	0,69
β globulin	(g/100 ml)	0,85	1,16	0,82
γ globulin	(g/100 ml)	1,21	1,64	1,55
Hb	(g/100 ml)	8,4		
Total W.C.C.	(mm^3)	4 700		
PMN	(%)	31		
Lymphocytes	(%)	66		
Monocytes	(%)	3		
Total lymph. count	(mm^3)	3102		

		Admission	2 weeks	6 weeks
IgG	(mg/100 ml)	1717	1533	1792
IgM	(mg/100 ml)	317	304	215
IgA	(mg/100 ml)	161	166	135
IgE	(u/ml)	1331	1086	553
C3	(mg/100 ml)	88	224	150

PHA Lymphocyte Transformation (dpm[^{14}C] -thymidine)

Lymph	Serum			
Control	AB	39238	33848	25163
Control	Patient	4305	32348	14531
Patient	AB	21686	25405	25012
Patient	Patient	3500	18067	14109

CASE No. 10

Diagnosis:	Kwashiorkor
Sex:	M
Age (months)	44
Weight (kg):	12,500
Expected weight for age (%):	78,6
Oedema:	++++
Dermatitis:	-
Hair changes:	-
Hepatomegaly:	1 cm
McLaren score:	9
Cultures: stool:	Trichuris trichuria, Ascaris lumbricoides, Giardia lamblia
Result:	Recovered

Laboratory Results:

		Admission	2 weeks	4 weeks
Total protein	(g/100 ml)	4,18	7,59	7,95
Albumin	(g/100 ml)	1,3	3,34	3,57
α_1 globulin	(g/100 ml)	0,34	0,21	0,23
α_2 globulin	(g/100 ml)	0,65	1,01	0,9
β globulin	(g/100 ml)	0,44	1,19	1,0
γ globulin	(g/100 ml)	1,45	1,83	2,27
Hb	(g/100 ml)	9,6		
Total W.C.C.	(mm^3)	7800		
PMN	(%)	54		
Lymphocytes	(%)	42		
Monocytes	(%)	1		

		Admission	2 weeks	4 weeks
Eosinophils	(%)	3		
Total lymph. count	(mm^3)	3276		
NBT (unstimulated)	(%)	41		
Blood group		0 +ve		
Anti-A agglutinins (titre)		1 : 128		
Anti-B agglutinins (titre)		1 : 16		
IgG	(mg/100 ml)	1710	1970	1720
IgM	(mg/100 ml)	165	148	158
IgA	(mg/100 ml)	145	162	173
IgE	(u/ml)	2810		3112
C3	(mg/100 ml)	52	181	202

PHA Lymphocyte Transformation (dpm $[^{14}C]$ -thymidine)

Lymph	Serum			
Control	AB	29674	32454	42027
Control	Patient	28756	25609	32228
Patient	AB	14354	20367	28779
Patient	Patient	3728	9079	14627

Mixed Lymphocyte Cultures (dpm $[^{14}C]$ -thymidine)

A = Patient B = Control

Lymph	Serum			
ABm	AB	1603	2258	1034
AmB	AB	1366	455	4768
AmBm	AB	19	37	14
AB	AB	2611	2264	4591
ABm	Patient	48	293	2599
AmB	Patient	65	114	4501
AmBm	Patient	-	46	-
AB	Patient	-	390	8005

CASE No. 11

Diagnosis:		Kwashiorkor
Sex:		F
Age (months):		48
Weight (kg):		10,050
Expected weight for age (%):		60,6
Oedema:		+++
Dermatitis:		-
Hair changes:		+
Hepatomegaly:		3 cm
McLaren score:		9
Cultures:	throat:	β haemolytic streptococci
	stool:	Salmonella
Result:		Recovered

Laboratory Results:

		Admission	2 weeks	4 weeks
Total protein	(g/100 ml)	4,68	7,19	7,94
Albumin	(g/100 ml)	1,9	2,94	3,57
α_1 globulin	(g/100 ml)	0,25	0,25	0,29
α_2 globulin	(g/100 ml)	0,71	1,53	1,02
β globulin	(g/100 ml)	0,58	1,18	1,27
γ globulin	(g/100 ml)	1,25	1,28	1,78
Hb	(g/100 ml)	10,2		
Total W.C.C.	(mm^3)	8 000		
PMN	(%)	57		
Lymphocytes	(%)	38		
Monocytes	(%)	2		

		Admission	2 weeks	4 weeks
Eosinophils	(%)	3		
Total lymph. count	(mm^3)	3040		
NBT (unstimulated)	(%)	44		
Blood group		A +ve		
Anti-B agglutinins (titre)		1 : 16		
IgG	(mg/100 ml)	1620	2260	2020
IgM	(mg/100 ml)	300	299	270
IgA	(mg/100 ml)	302	254	285
IgE	(u/ml)	936	536	536
C3	(mg/100 ml)	43	197	175

PHA Lymphocyte Transformation (dpm $[^{14}C]$ -thymidine)

Lymph	Serum			
Control	AB	19305	26892	11387
Control	Patient	6591	19568	9706
Patient	AB	26762	20999	23656
Patient	Patient	6002	6124	22338

Mixed Lymphocyte Cultures (dpm $[^{14}C]$ -thymidine)

A = Patient B = Control

Lymph	Serum			
ABm	AB	5384	7084	3928
AmB	AB	1755	2545	2762
AmBm	AB	17	63	21
AB	AB	11760	4257	2288
ABm	Patient	-	7568	9073
AmB	Patient	226	3251	5847
AmBm	Patient	-	44	-
AB	Patient	4526	3866	5985

CASE No. 12

Diagnosis:	Kwashiorkor and Measles
Sex:	F
Age (months):	10
Weight (kg):	6,710
Expected weight for age (%):	73,2
Oedema:	++++
Dermatitis:	+
Hair changes:	+
Hepatomegaly:	3 cm
McLaren score:	11
Cultures: skin:	Staphylococcus aureus and β haemolytic streptococci
Result:	Developed measles 12 days after admission. Transferred to City Hospital for Infectious Diseases.

Laboratory Results:

		Admission
Total protein	(g/100 ml)	4,74
Albumin	(g/100 ml)	2,21
α_1 globulin	(g/100 ml)	0,33
α_2 globulin	(g/100 ml)	0,74
β globulin	(g/100 ml)	0,60
γ globulin	(g/100 ml)	0,87
Hb	(g/100 ml)	8,9
Total W.C.C.	(mm^3)	20 200
PMN	(%)	58

		Admission
Lymphocytes	(%)	32
Monocytes	(%)	7
Eosinophils	(%)	1
Total lymph. count	(mm^3)	6464
NBT (unstimulated)	(%)	26
Blood group		O +ve
Anti-A agglutinins (titre)		1 : 32
Anti-B agglutinins (titre)		1 : 32
IgG	(mg/100 ml)	858
IgM	(mg/100 ml)	142
IgA	(mg/100 ml)	82
IgE	(u/ml)	49
C3	(mg/100 ml)	96

PHA Lymphocyte Transformation (dpm $[^{14}C]$ -thymidine)

Lymph	Serum	
Control	AB	29756
Control	Patient	15927
Patient	AB	19909
Patient	Patient	4243

Mixed Lymphocyte Cultures (dpm $[^{14}C]$ -thymidine)

A = Patient B = Control

Lymph	Serum	
ABm	AB	2135
AmB	AB	2917
AmBm	AB	8
AB	AB	3836

		Admission
Lymph	Serum	
ABm	Patient	636
AmB	Patient	255
AmBm	Patient	8
AB	Patient	1828

CASE No. 13

Diagnosis:		Kwashiorkor, Herpes stomatitis, Pneumonia and Otitis media
Sex:		F
Age (months):		17
Weight (kg):		7,760
Expected weight for age (%):		72
Oedema:		+++
Dermatitis:		+
Hair changes:		+
Hepatomegaly:		6 cm
McLaren score:		13
Cultures:	skin:	E. coli
Chest X-ray:		Patchy pneumonitis
Result:		Died 2 days after admission. Post mortem findings included Herpes stomatitis, pneumonia and haemorrhagic ulceration of the colonic mucosa.

Laboratory Results:

		Admission
Total protein	(g/100 ml)	3,1
Albumin	(g/100 ml)	1,11
α_1 globulin	(g/100 ml)	0,45
α_2 globulin	(g/100 ml)	0,64
β globulin	(g/100 ml)	0,27
γ globulin	(g/100 ml)	0,65
Hb	(g/100 ml)	9,1

		Admission
Total W.C.C.	(mm^3)	11 400
PMN	(%)	70
Lymphocytes	(%)	28
Monocytes	(%)	0
Eosinophils	(%)	2
Total lymph. count	(mm^3)	3192
NBT (unstimulated)	(%)	10
(stimulated)	(%)	28
Blood group		0 +ve
Anti-A agglutinins (titre)		1 : 64
Anti-B agglutinins (titre)		1 : 64
IgG	(mg/100 ml)	920
IgM	(mg/100 ml)	124
IgA	(mg/100 ml)	97

PHA Lymphocyte Transformation (dpm $[^{14}C]$ -thymidine)

Lymph	Serum	
Control	AB	15455
Control	Patient	1599
Patient	AB	36292
Patient	Patient	421
T cells (%)	Patient	47
	Control	65
B cells (%)	Patient	45
	Control	24

CASE No. 14

Diagnosis:	Kwashiorkor with Pneumonia and Otitis media
Sex:	M
Age (months):	18
Weight (kg):	7 060
Expected weight for age (%):	64
Oedema:	+++
Dermatitis:	++
Hair changes:	-
Hepatomegaly:	2 cm
McLaren score:	12
Cultures: stool:	Candida albicans
Chest X-ray:	Right upper lobe pneumonia
Result:	Recovered

Laboratory Results:

		Admission	4 weeks
Total protein	(g/100 ml)	5,0	
Albumin	(g/100 ml)	1,91	
α_1 globulin	(g/100 ml)	0,2	
α_2 globulin	(g/100 ml)	0,57	
β globulin	(g/100 ml)	0,48	
γ globulin	(g/100 ml)	1,84	
Hb	(g/100 ml)	9,9	11,1
Total W.C.C.	(mm^3)	13 700	16 100
PMN	(%)	78	43
Lymphocytes	(%)	20	54
Monocytes	(%)	2	2

		Admission	4 weeks
Total lymph. count	(mm^3)	2740	
NBT (unstimulated)	(%)	27	
Blood group		A +ve	
Anti-B agglutinins (titre)		1 : 64	1 : 256
IgG	(mg/100 ml)	1720	2812
IgM	(mg/100 ml)	235	400
IgA	(mg/100 ml)	216	264
IgE	(u/ml)		42
C3	(mg/100 ml)	42	

PHA Lymphocyte Transformation (dpm $[^{14}C]$ -thymidine)

Lymph	Serum		
Control	AB	23213	
Control	Patient	15821	
Patient	AB	39593	
Patient	Patient	42256	
T cells (%)	Patient	72	70
	Control	66	64
B cells (%)	Patient	31	37
	Control	40	33

CASE No. 15

Diagnosis:	Kwashiorkor and Otitis media
Sex:	F
Age (months):	19
Weight (kg):	7,350
Expected weight for age (%):	65%
Oedema:	+++
Dermatitis:	++
Hair changes:	+
Hepatomegaly:	2 cm
McLaren score:	10
Cultures: throat:	β haemolytic streptococci
Result:	Recovered

Laboratory Results:

		Admission	4 weeks
Total protein	(g/100 ml)	5,8	7,7
Albumin	(g/100 ml)	2,82	3,94
α_1 globulin	(g/100 ml)	0,28	0,18
α_2 globulin	(g/100 ml)	0,74	0,97
β globulin	(g/100 ml)	0,68	1,08
γ globulin	(g/100 ml)	1,29	1,53
Hb	(g/100 ml)	7,9	8,3
Total W.C.C.	(mm^3)	9 300	13 400
PMN	(%)	31	34
Lymphocytes	(%)	61	58
Monocytes	(%)	6	3
Eosinophils	(%)	2	-

		Admission	4 weeks
Total lymph. count	(mm^3)	5673	-
NBT (unstimulated)	(%)	14	
Blood group		B +ve	
Anti-A agglutinin (titre)		1 : 4	1 : 32
IgG	(mg/100 ml)	1650	1590
IgM	(mg/100 ml)	99	136
IgA	(mg/100 ml)	102	71
IgE	(u/ml)		776
C3	(mg/100 ml)	100	115

PHA Lymphocyte Transformation (dpm [^{14}C] -thymidine)

Lymph	Serum	
Control	AB	43109
Control	Patient	25054
Patient	AB	3271
Patient	Patient	1843
T cells (%)	Patient	48
	Control	75
B cells (%)	Patient	54
	Control	31

CASE No. 16

Diagnosis:		Marasmic Kwashiorkor with Anaemia
Sex:		M
Age (months):		22
Weight (kg):		6,970
Expected weight for age (%):		57
Oedema:		++
Dermatitis:		-
Hair changes:		+
Hepatomegaly:		4 cm
McLaren score:		10
Cultures:	stool:	Shigella
	skin:	Staphylococci, Klebsiella and Enterococci
	throat:	Candida albicans
Result:		Recovered

Laboratory Results:

		Admission	4 weeks
Total protein	(g/100 ml)	5,9	7,7
Albumin	(g/100 ml)	1,82	3,56
α_1 globulin	(g/100 ml)	0,44	0,21
α_2 globulin	(g/100 ml)	1,02	0,95
β globulin	(g/100 ml)	0,66	0,88
γ globulin	(g/100 ml)	1,97	2,10
Hb	(g/100 ml)	3,4	12,9
Total W.C.C.	(mm^3)	16 800	11 100
PMN	(%)	45	29
Lymphocytes	(%)	56	64

		Admission	4 weeks
Monocytes	(%)	2	4
Total lymph. count	(mm^3)	9408	
NBT (unstimulated)	(%)	36	44
Blood group		0 +ve	
Anti-A agglutinins (titre)		1 : 32	1 : 64
Anti-B agglutinins (titre)		1 : 32	1 : 128
IgG	(mg/100 ml)	1180	2380
IgM	(mg/100 ml)	165	184
IgA	(mg/100 ml)	202	92
IgE	(u/ml)	2330	2236
C3	(mg/100 ml)	50	

PHA Lymphocyte Transformation (dpm $[^{14}C]$ -thymidine)

Lymph	Serum		
Control	AB	9374	
Control	Patient	6291	
Patient	AB	67585	
Patient	Patient	41121	
T cells (%)	Patient	67	55
	Control	66	60
B cells (%)	Patient	18	39
	Control	24	33

CASE No. 17

Diagnosis:	Marasmic Kwashiorkor and Pneumonia
Sex:	F
Age (months):	26
Weight (kg):	6,130
Expected weight for age (%):	48%
Oedema:	++
Dermatitis:	+
Hair changes:	+
Hepatomegaly:	2 cm
McLaren score:	13
Cultures: skin:	Staphylococci and Streptococci
Chest X-ray:	Right lower lobe pneumonia
Result:	Died 3 days after admission. No post mortem.

Laboratory Results:

		Admission
Total protein	(g/100 ml)	2,9
Albumin	(g/100 ml)	1,43
α_1 globulin	(g/100 ml)	0,20
α_2 globulin	(g/100 ml)	0,33
β globulin	(g/100 ml)	0,30
γ globulin	(g/100 ml)	0,64
Hb	(g/100 ml)	9,8
Total W.C.C.	(mm^3)	12 400
PMN	(%)	73
Lymphocytes	(%)	26
Monocytes	(%)	1

		Admission
Total lymph. count	(mm^3)	3224
NBT (unstimulated)	(%)	75
Blood group		A +ve
Anti-B agglutinins (titre)		1 : 64
C3	(mg/100 ml)	27

PHA Lymphocyte Transformation (dpm $[^{14}C]$ -thymidine)

Lymph	Serum	
Control	AB	28876
Control	Patient	6754
Patient	AB	8931
Patient	Patient	2571
T cells (%)	Patient	32
	Control	56
B cells (%)	Patient	35
	Control	25

CASE No. 18

Diagnosis:	Kwashiorkor and Pneumonia
Sex:	M
Age (months):	26
Weight (kg):	8,380
Expected weight for age (%):	65
Oedema:	+++
Dermatitis:	++
Hair changes:	+
Hepatomegaly:	1 cm
McLaren score:	13
Cultures: stool:	Candida albicans
skin:	Staphylococci and Streptococci
throat:	Candida albicans and Staphylococci
Chest X-ray:	Bilateral bronchopneumonia
Result:	Died 4 days after admission. Post mortem showed extensive bronchopneumonia.

Laboratory Results:

		Admission
Total protein	(g/100 ml)	2,9
Albumin	(g/100 ml)	1,06
α_1 globulin	(g/100 ml)	0,21
α_2 globulin	(g/100 ml)	0,44
β globulin	(g/100 ml)	0,38
γ globulin	(g/100 ml)	0,81
Hb	(g/100 ml)	6,8
Total W.C.C.	(mm^3)	10 900
PMN	(%)	95

		Admission
Lymphocytes	(%)	5
Total lymph. count	(mm^3)	545
NBT (unstimulated)	(%)	18
Blood group		0 +ve
Anti-A agglutinins (titre)		1 : 16
Anti-B agglutinins (titre)		1 : 32
IgG	(mg/100 ml)	980
IgM	(mg/100 ml)	70
IgA	(mg/100 ml)	175
C3	(mg/100 ml)	27

PHA Lymphocyte Transformation (dpm $[^{14}C]$ -thymidine)

Lymph	Serum	
Control	AB	20949
Control	Patient	1267
Patient	AB	5342
Patient	Patient	217
T cells (%)	Patient	28
	Control	70
B cells (%)	Patient	68
	Control	34

CASE No. 19

Diagnosis:		Kwashiorkor
Sex:		F
Age (months):		55
Weight (kg):		10,850
Expected weight for age (%):		63
Oedema:		+
Dermatitis:		+
Hair changes:		+
Hepatomegaly:		6 cm
McLaren score:		13
Cultures:	stool:	Candida albicans
	skin:	Staphylococci, Klebsiella and Pseudomonas
Result:		Recovered

Laboratory Results:

		Admission	2 weeks	4 weeks
Total protein	(g/100 ml)	3,1		7,0
Albumin	(g/100 ml)	1,27		3,57
α_1 globulin	(g/100 ml)	0,19		0,19
α_2 globulin	(g/100 ml)	0,38		0,77
β globulin	(g/100 ml)	0,34		0,88
γ globulin	(g/100 ml)	0,92		1,59
Hb	(g/100 ml)	9,5	8,3	
Total W.C.C.	(mm^3)	15 900	9 100	
PMN	(%)	56	45	
Lymphocytes	(%)	44	52	
Monocytes	(%)	-	3	

		Admission	2 weeks	4 weeks
Total lymph. count	(mm^3)	6996		
NBT (unstimulated)	(%)	16		
Blood group		0 +ve		
Anti-A agglutinins (titre)		1 : 512		1 : 64
Anti-B agglutinins (titre)		1 : 32		1 : 64
IgG	(mg/100 ml)	1200		
IgM	(mg/100 ml)	138		
IgA	(mg/100 ml)	173		
IgE	(u/ml)	113		
C3	(mg/100 ml)	26		115

PHA Lymphocyte Transformation (dpm $[^{14}C]$ -thymidine)

Lymph	Serum		
Control	AB	17629	49402
Control	Patient	4597	50264
Patient	AB	16809	176614
Patient	Patient	7164	226297
T cells (%)	Patient	68	50
	Control	50	33
B cells (%)	Patient	22	13
	Control	22	30

CASE No. 20

Diagnosis:	Kwashiorkor and Pneumonia
Sex:	F
Age (months):	12
Weight (kg):	5,980
Expected weight for age (%):	62
Oedema:	+++
Dermatitis:	+++
Hair changes:	-
Hepatomegaly:	-
McLaren score:	12
Cultures: stool:	Salmonella
Chest X-ray:	Bilateral pneumonitis
Result:	Recovered

Laboratory Results:

		Admission
Total protein	(g/100 ml)	2,78
Albumin	(g/100 ml)	1,18
α_1 globulin	(g/100 ml)	0,22
α_2 globulin	(g/100 ml)	0,50
β globulin	(g/100 ml)	0,36
γ globulin	(g/100 ml)	0,52
Hb	(g/100 ml)	8,4
Total W.C.C.	(mm^3)	17 000
PMN	(%)	41
Lymphocytes	(%)	51
Monocytes	(%)	8

		Admission
Total lymph. count	(mm^3)	6827
IgG	(mg/100 ml)	488
IgM	(mg/100 ml)	63
IgA	(mg/100 ml)	53
IgE	(u/ml)	18
C3	(mg/100 ml)	73

PHA Lymphocyte Transformation (dpm $[^{14}C]$ -thymidine)

Lymph	Serum	
Control	AB	14934
Control	Patient	5997

CASE No. 21

Diagnosis:		Kwashiorkor and Pneumonia
Sex:		F
Age (months):		12
Weight (kg):		7,800
Expected weight for age (%):		80
Oedema:		+++
Dermatitis:		++
Hair changes:		-
Hepatomegaly:		1 cm
McLaren score:		11
Cultures:	throat:	β haemolytic streptococci
Chest X-ray:		Right upper lobe pneumonia
Result:		Recovered

Laboratory Results:

		Admission
Total protein	(g/100 ml)	4,22
Albumin	(g/100 ml)	1,55
α_1 globulin	(g/100 ml)	0,23
α_2 globulin	(g/100 ml)	0,52
β globulin	(g/100 ml)	0,56
γ globulin	(g/100 ml)	1,36
IgG	(mg/100 ml)	1768
IgM	(mg/100 ml)	293
IgA	(mg/100 ml)	180
C3	(mg/100 ml)	59

PHA Lymphocyte Transformation (dpm $[^{14}C]$ -thymidine)

		Admission
Lymph	Serum	
Control	AB	34903
Control	Patient	9428

CASE No. 22

Diagnosis:	Kwashiorkor
Sex:	M
Age (months):	13
Weight (kg):	7,890
Expected weight for age (%):	77,2
Oedema:	+++
Dermatitis:	+
Hair changes:	+
Hepatomegaly:	2 cm
McLaren score:	11
Result:	Recovered

Laboratory Results:

		Admission
Total protein	(g/100 ml)	3,97
Albumin	(g/100 ml)	2,12
α_1 globulin	(g/100 ml)	0,21
α_2 globulin	(g/100 ml)	0,62
β globulin	(g/100 ml)	0,36
γ globulin	(g/100 ml)	0,66
Hb	(g/100 ml)	8,9
Total W.C.C.	(mm^3)	11 800
IgG	(mg/100 ml)	416
IgM	(mg/100 ml)	117
IgA	(mg/100 ml)	54
IgE	(u/ml)	1953
C3	(mg/100 ml)	65

PHA Lymphocyte Transformation (dpm $[^{14}C]$ -thymidine)

		Admission
Lymph	Serum	
Control	AB	10300
Control	Patient	5769

CASE No. 23

Diagnosis:	Kwashiorkor
Sex:	F
Age (months):	14
Weight (kg):	7,135
Expected weight for age (%):	70,5
Oedema:	+++
Dermatitis:	++
Hair changes:	-
Hepatomegaly:	-
McLaren score:	12
Result:	Recovered

Laboratory Results:

		Admission
Total protein	(g/100 ml)	4,48
Albumin	(g/100 ml)	1,49
α_1 globulin	(g/100 ml)	0,19
α_2 globulin	(g/100 ml)	0,59
β globulin	(g/100 ml)	0,62
γ globulin	(g/100 ml)	1,59
Hb	(g/100 ml)	8,4
Total W.C.C.	(mm^3)	8 900
IgG	(mg/100 ml)	1768
IgM	(mg/100 ml)	219
IgA	(mg/100 ml)	269
IgE	(u/ml)	58

PHA Lymphocyte Transformation (dpm $[^{14}C]$ -thymidine)

		Admission
Lymph	Serum	
Control	AB	25763
Control	Patient	20524

CASE No. 24

Diagnosis:	Kwashiorkor and Scabies
Sex:	F
Age (months):	15
Weight (kg):	7,490
Expected weight for age (%):	72,4
Oedema:	+
Dermatitis:	+
Hair changes:	-
Hepatomegaly:	2 cm
McLaren score:	11
Cultures: eye:	Staphylococci and Streptococci
Result:	Recovered

Laboratory Results:

		Admission
Total protein	(g/100 ml)	3,81
Albumin	(g/100 ml)	1,59
α_1 globulin	(g/100 ml)	0,24
α_2 globulin	(g/100 ml)	0,57
β globulin	(g/100 ml)	0,49
γ globulin	(g/100 ml)	0,91
Hb	(g/100 ml)	9,8
Total W.C.C.	(mm^3)	11 700
PMN	(%)	67
Lymphocytes	(%)	27
Monocytes	(%)	6
Total lymph. count	(mm^3)	3159

		Admission
IgG	(mg/100 ml)	1378
IgM	(mg/100 ml)	98
IgA	(mg/100 ml)	126
IgE	(u/ml)	133
C3	(mg/100 ml)	73

PHA Lymphocyte Transformation (dpm $[^{14}C]$ -thymidine)

Lymph	Serum	
Control	AB	10300
Control	Patient	5784

CASE No. 25

Diagnosis:	Kwashiorkor and Pneumonia
Sex:	M
Age (months):	15
Weight (kg):	6,850
Expected weight for age (%):	64,2
Oedema:	+++
Dermatitis:	++
Hair changes:	-
Hepatomegaly:	1,5 cm
McLaren score:	10
Cultures: stool:	Salmonella
Chest X-ray:	Bilateral pneumonitis
Result:	Recovered

Laboratory Results:

		Admission
Total protein	(g/100 ml)	4,81
Albumin	(g/100 ml)	2,17
α_1 globulin	(g/100 ml)	0,27
α_2 globulin	(g/100 ml)	0,68
β globulin	(g/100 ml)	0,55
γ globulin	(g/100 ml)	1,14
Hb	(g/100 ml)	9,5
Total W.C.C.	(mm^3)	7 700
PMN	(%)	57
Lymphocytes	(%)	32
Monocytes	(%)	11

		Admission
Total lymph. count	(mm^3)	2464
IgG	(mg/100 ml)	898
IgM	(mg/100 ml)	235
IgA	(mg/100 ml)	86
IgE	(u/ml)	98
C3	(mg/100 ml)	81

PHA Lymphocyte Transformation (dpm $[^{14}C]$ -thymidine)

Lymph	Serum	
Control	AB	9122
Control	Patient	5240

CASE No. 26

Diagnosis:	Marasmic Kwashiorkor
Sex:	M
Age (months):	17
Weight (kg):	5,940
Expected weight for age (%):	53,5
Oedema:	+++
Dermatitis:	-
Hair changes:	-
Hepatomegaly:	-
McLaren score:	8
Cultures: stool:	Giardia lamblia
Result:	Recovered

Laboratory Results:

		Admission
Total protein	(g/100 ml)	4,68
Albumin	(g/100 ml)	1,98
α_1 globulin	(g/100 ml)	0,43
α_2 globulin	(g/100 ml)	0,95
β globulin	(g/100 ml)	0,40
γ globulin	(g/100 ml)	0,92
Hb	(g/100 ml)	11,1
Total W.C.C.	(mm^3)	12 300
PMN	(%)	43
Lymphocytes	(%)	52
Monocytes	(%)	5

		Admission
Total lymph. count	(mm^3)	6396
IgG	(mg/100 ml)	931
IgM	(mg/100 ml)	104
IgA	(mg/100 ml)	49
IgE	(u/ml)	117
C3	(mg/100 ml)	83

PHA Lymphocyte Transformation (dpm $[^{14}C]$ -thymidine)

Lymph	Serum	
Control	AB	11660
Control	Patient	4923

CASE No. 27

Diagnosis:	Kwashiorkor
Sex:	F
Age (months):	18
Weight (kg):	8,900
Expected weight for age (%):	80
Oedema:	++++
Dermatitis:	-
Hair changes:	+
Hepatomegaly:	2 cm
McLaren score:	9
Cultures: stool:	Giardia lamblia
Result:	Recovered

Laboratory Results:

		Admission
Total protein	(g/100 ml)	4,42
Albumin	(g/100 ml)	1,7
α_1 globulin	(g/100 ml)	0,29
α_2 globulin	(g/100 ml)	0,85
β globulin	(g/100 ml)	0,62
γ globulin	(g/100 ml)	0,96
Hb	(g/100 ml)	10,6
Total W.C.C.	(mm^3)	19 300
PMN	(%)	80
Lymphocytes	(%)	18
Monocytes	(%)	1
Eosinophils	(%)	1

		Admission
Total lymph. count	(mm^3)	3474
IgG	(mg/100 ml)	780
IgM	(mg/100 ml)	228
IgA	(mg/100 ml)	140
C3	(mg/100 ml)	68

PHA Lymphocyte Transformation (dpm $[^{14}C]$ -thymidine)

Lymph	Serum	
Control	AB	31271
Control	Patient	16210

CASE No. 28

Diagnosis:	Marasmic Kwashiorkor and Pneumonia
Sex:	M
Age (months):	19
Weight (kg):	5,340
Expected weight for age (%):	46,3
Oedema:	+++
Dermatitis:	-
Hair changes:	+
Hepatomegaly:	3 cm
McLaren score:	9
Chest X-ray:	Bilateral bronchopneumonia
Result:	Recovered

Laboratory Results:

		Admission
Total protein	(g/100 ml)	4,85
Albumin	(g/100 ml)	1,57
α_1 globulin	(g/100 ml)	0,37
α_2 globulin	(g/100 ml)	1,08
β globulin	(g/100 ml)	0,81
γ globulin	(g/100 ml)	1,02
Hb	(g/100 ml)	7,5
Total W.C.C.	(mm^3)	12 100
PMN	(%)	43
Lymphocytes	(%)	51
Monocytes	(%)	5
Eosinophils	(%)	1

		Admission
Total lymph. count	(mm^3)	6426
IgG	(mg/100 ml)	1128
IgM	(mg/100 ml)	165
IgA	(mg/100 ml)	170
IgE	(u/ml)	1329
C3	(mg/100 ml)	132

PHA Lymphocyte Transformation (dpm $[^{14}C]$ -thymidine)

Lymph	Serum	
Control	AB	14832
Control	Patient	3897

CASE No. 29

Diagnosis:		Marasmic Kwashiorkor and Anaemia
Sex:		M
Age (months):		22
Weight (kg):		7,4
Expected weight for age (%):		60
Oedema:		+++
Dermatitis:		-
Hair changes		-
Hepatomegaly:		-
McLaren score:		8
Cultures:	stool:	Shigella and Giardia lamblia
	skin:	Staphylococcus aureus
Result:		Recovered

Laboratory Results:

		Admission
Total protein	(g/100 ml)	3,57
Albumin	(g/100 ml)	1,15
α_1 globulin	(g/100 ml)	0,33
α_2 globulin	(g/100 ml)	0,53
β globulin	(g/100 ml)	0,52
γ globulin	(g/100 ml)	1,05
Hb	(g/100 ml)	5,6
Total W.C.C.	(mm^3)	12 200
PMN	(%)	80
Lymphocytes	(%)	15
Monocytes	(%)	5

		Admission
Total lymph. count	(mm^3)	1830
IgG	(mg/100 ml)	563
IgM	(mg/100 ml)	183
IgA	(mg/100 ml)	137
IgE	(u/ml)	27

PHA Lymphocyte Transformation (dpm $[^{14}C]$ -thymidine)

Lymph	Serum	
Control	AB	5536
Control	Patient	2222

CASE No. 30

Diagnosis:	Kwashiorkor and Pneumonia
Sex:	F
Age (months):	22
Weight (kg):	9,500
Expected weight for age (%):	79,9
Oedema:	+++
Dermatitis:	-
Hair changes:	-
Hepatomegaly:	3 cm
McLaren score:	9
Chest X-ray:	Bilateral lobar pneumonia
Result:	Unknown (Absconded)

Laboratory Results:

		Admission
Total protein	(g/100 ml)	4,03
Albumin	(g/100 ml)	1,62
α_1 globulin	(g/100 ml)	0,2
α_2 globulin	(g/100 ml)	0,57
β globulin	(g/100 ml)	0,59
γ globulin	(g/100 ml)	1,06
Hb	(g/100 ml)	8,1
Total W.C.C.	(mm^3)	9 300
IgE	(u/ml)	12

PHA Lymphocyte Transformation (dpm $[^{14}C]$ -thymidine)

		Admission
Lymph	Serum	
Control	AB	14881
Control	Patient	6389

CASE No. 31

Diagnosis:	Marasmic Kwashiorkor
Sex:	F
Age (months):	23
Weight (kg):	6,880
Expected weight for age (%):	56,9
Oedema:	+++
Dermatitis:	-
Hair changes:	-
Hepatomegaly:	-
McLaren score:	9
Result:	Recovered

Laboratory Results:

		Admission
Total protein	(g/100 ml)	4,15
Albumin	(g/100 ml)	1,27
α_1 globulin	(g/100 ml)	0,34
α_2 globulin	(g/100 ml)	0,58
β globulin	(g/100 ml)	0,68
γ globulin	(g/100 ml)	1,29
Hb	(g/100 ml)	9,8
Total W.C.C.	(mm^3)	10 200
PMN	(%)	56
Lymphocytes	(%)	38
Monocytes	(%)	3
Eosinophils	(%)	1
Total lymph. count	(mm^3)	3876

		Admission
IgG	(mg/100 ml)	1186
IgM	(mg/100 ml)	227
IgA	(mg/100 ml)	231
IgE	(u/ml)	685
C3	(mg/100 ml)	66

PHA Lymphocyte Transformation (dpm $[^{14}C]$ -thymidine)

Lymph	Serum	
Control	AB	8807
Control	Patient	5858

CASE No. 32

Diagnosis:	Kwashiorkor, Otitis media and Pneumonia
Sex:	M
Age (months):	24
Weight (kg):	7,950
Expected weight for age (%):	63,3
Oedema:	+++
Dermatitis:	++
Hair changes:	-
Hepatomegaly:	1 cm
McLaren score:	13
Chest X-ray:	Bilateral bronchopneumonia
Result:	Recovered

Laboratory Results:

		Admission
Total protein	(g/100 ml)	3,61
Albumin	(g/100 ml)	1,47
α_1 globulin	(g/100 ml)	0,26
α_2 globulin	(g/100 ml)	0,73
β globulin	(g/100 ml)	0,48
γ globulin	(g/100 ml)	0,66
Hb	(g/100 ml)	11,3
Total W.C.C.	(mm^3)	28 400
PMN	(%)	58
Lymphocytes	(%)	39
Monocytes	(%)	3
Total lymph. count	(mm^3)	11 076

		Admission
IgG	(mg/100 ml)	531
IgM	(mg/100 ml)	122
IgA	(mg/100 ml)	91
IgE	(u/ml)	31
C3	(mg/100 ml)	54

PHA Lymphocyte Transformation (dpm $[^{14}C]$ -thymidine)

Lymph	Serum	
Control	AB	27856
Control	Patient	17714

CASE No. 33

Diagnosis:	Kwashiorkor
Sex:	F
Age (months):	28
Weight (kg):	10,580
Expected weight for age (%):	80
Oedema:	++
Dermatitis:	++
Hair changes:	-
Hepatomegaly:	3 cm
McLaren score:	13
Cultures: stool:	Salmonella, Giardia lamblia and Ascaris lumbricoides
Result:	Recovered

Laboratory Results:

		Admission
Total protein	(g/100 ml)	3,92
Albumin	(g/100 ml)	1,31
α_1 globulin	(g/100 ml)	0,29
α_2 globulin	(g/100 ml)	0,64
β globulin	(g/100 ml)	0,54
γ globulin	(g/100 ml)	1,13
Hb	(g/100 ml)	8,2
Total W.C.C.	(mm^3)	10 400
PMN	(%)	74
Lymphocytes	(%)	23
Monocytes	(%)	3

		Admission
Total lymph. count	(mm^3)	2080
IgG	(mg/100 ml)	968
IgM	(mg/100 ml)	165
IgA	(mg/100 ml)	121
IgE	(u/ml)	471
C3	(mg/100 ml)	90

PHA Lymphocyte Transformation (dpm $[^{14}C]$ -thymidine)

Lymph	Serum	
Control	AB	18917
Control	Patient	7142

CASE No. 34

Diagnosis:	Kwashiorkor
Sex:	F
Age (months):	30
Weight (kg):	8,800
Expected weight for age (%):	65,4
Oedema:	++++
Dermatitis:	+++
Hair changes:	-
Hepatomegaly:	3 cm
McLaren score:	15
Result:	Recovered

Laboratory Results:

		Admission
Total protein	(g/100 ml)	3,98
Albumin	(g/100 ml)	0,95
α_1 globulin	(g/100 ml)	0,19
α_2 globulin	(g/100 ml)	0,66
β globulin	(g/100 ml)	0,45
γ globulin	(g/100 ml)	1,73
Hb	(g/100 ml)	7,5
Total W.C.C.	(mm^3)	14 900
PMN	(%)	74
Lymphocytes	(%)	24
Monocytes	(%)	2
Total lymph. count	(mm^3)	3576

		Admission
IgG	(mg/100 ml)	2202
IgM	(mg/100 ml)	250
IgA	(mg/100 ml)	245
C3	(mg/100 ml)	70

PHA Lymphocyte Transformation (dpm $[^{14}C]$ -thymidine)

Lymph	Serum	
Control	AB	34903
Control	Patient	8479

CASE No. 35

Diagnosis:		Kwashiorkor
Sex:		M
Age (months):		41
Weight (kg):		11,510
Expected weight for age (%):		74,4
Oedema:		++
Dermatitis:		++
Hair changes:		-
Hepatomegaly:		-
McLaren score:		12
Cultures:	stool:	Salmonella
	skin:	Staphylococcus
Result:		Recovered

Laboratory Results:

		Admission
Total protein	(g/100 ml)	3,45
Albumin	(g/100 ml)	1,32
α_1 globulin	(g/100 ml)	0,26
α_2 globulin	(g/100 ml)	0,66
β globulin	(g/100 ml)	0,56
γ globulin	(g/100 ml)	0,66
Hb	(g/100 ml)	12,9
Total W.C.C.	(mm^3)	20 200
PMN	(%)	60
Lymphocytes	(%)	36
Monocytes	(%)	4

		Admission
Total lymph. count	(mm^3)	7272
IgG	(mg/100 ml)	553
IgM	(mg/100 ml)	129
IgA	(mg/100 ml)	69
IgE	(u/ml)	896
C3	(mg/100 ml)	74,8

PHA Lymphocyte Transformation (dpm $[^{14}C]$ -thymidine)

Lymph	Serum	
Control	AB	6043
Control	Patient	2429

CASE No. 36

Diagnosis:	Kwashiorkor and Pneumonia
Sex:	F
Age (months)	50
Weight (kg):	11,500
Expected weight for age (%):	69,4
Oedema:	++
Dermatitis:	+
Hair changes:	-
Hepatomegaly:	-
McLaren score:	12
Cultures: stool:	E. coli 0127, Trichuris trichuria
Chest X-ray :	Bilateral bronchopneumonia
Result:	Recovered

Laboratory Results:

		Admission
Total protein	(g/100 ml)	3,3
Albumin	(g/100 ml)	1,08
α_1 globulin	(g/100 ml)	0,26
α_2 globulin	(g/100 ml)	0,54
β globulin	(g/100 ml)	0,64
γ globulin	(g/100 ml)	0,78
Hb	(g/100 ml)	8,3
Total W.C.C.	(mm^3)	9 200
PMN	(%)	61
Lymphocytes	(%)	37
Monocytes	(%)	1

		Admission
Eosinophils	(%)	1
Total lymph. count	(mm^3)	3404
IgG	(mg/100 ml)	853
IgM	(mg/100 ml)	74
IgA	(mg/100 ml)	75
IgE	(u/ml)	288
C3	(mg/100 ml)	74

PHA Lymphocyte Transformation (dpm $[^{14}C]$ -thymidine)

Lymph	Serum	
Control	AB	11660
Control	Patient	6871

A. 2 PAEDIATRIC CONTROLS

CONTROL No. 1

Sex:	F
Age (months):	11
Weight (kg):	11,000
Expected weight for age (%):	117

Laboratory Results:

Total protein	(g/100 ml)	6,74
Albumin	(g/100 ml)	3,96
α_1 globulin	(g/100 ml)	0,22
α_2 globulin	(g/100 ml)	0,78
β globulin	(g/100 ml)	0,93
γ globulin	(g/100 ml)	0,85
IgG	(mg/100 ml)	695
IgM	(mg/100 ml)	83
IgA	(mg/100 ml)	72
C3	(mg/100 ml)	123

PHA Lymphocyte Transformation (dpm $[^{14}C]$ 1thymidine)

Lymph	Serum	
Adult Reference	AB	30326
Adult Reference	Paediatric Control	22319
Paediatric Control	AB	55180
Paediatric Control	Paediatric Control	50822

CONTROL No. 2

Sex:	F
Age (months):	12
Weight (kg):	10,100
Expected weight for age (%):	104,8

Laboratory Results:

Total protein	(g/100 ml)	6,59
Albumin	(g/100 ml)	4,16
α_1 globulin	(g/100 ml)	0,21
α_2 globulin	(g/100 ml)	0,79
β globulin	(g/100 ml)	0,82
γ globulin	(g/100 ml)	0,61
IgG	(mg/100 ml)	474
IgM	(mg/100 ml)	96
IgA	(mg/100 ml)	134
C3	(mg/100 ml)	149

PHA Lymphocyte Transformation (dpm $[^{14}C]$ -thymidine)

Lymph	Serum	
Adult Reference	AB	33169
Adult Reference	Paediatric Control	25136
Paediatric Control	AB	46410
Paediatric Control	Paediatric Control	36129

CONTROL No. 3

Sex:	F
Age (months):	13
Weight (kg):	10,900
Expected weight for age (%)	113,1

Laboratory Results:

Total protein	(g/100 ml)	6,50
Albumin	(g/100 ml)	3,96
α_1 globulin	(g/100 ml)	0,27
α_2 globulin	(g/100 ml)	0,88
β globulin	(g/100 ml)	0,74
γ globulin	(g/100 ml)	0,64
IgG	(mg/100 ml)	570
IgM	(mg/100 ml)	66
IgA	(mg/100 ml)	28
IgE	(u/ml)	nil
C3	(mg/100 ml)	237

PHA Lymphocyte Transformation (dpm $[^{14}C]$ -thymidine)

Lymph	Serum	
Adult Reference	AB	32606
Adult Reference	Paediatric Control	24651
Paediatric Control	AB	25573
Paediatric Control	Paediatric Control	28020

CONTROL No. 4

Sex:	M
Age (months):	14
Weight (kg):	11,030
Expected weight for age (%):	105,7

Laboratory Results:

Total protein	(g/100 ml)	7,08
Albumin	(g/100 ml)	4,21
α_1 globulin	(g/100 ml)	0,24
α_2 globulin	(g/100 ml)	0,73
β globulin	(g/100 ml)	0,86
γ globulin	(g/100 ml)	1,04
IgG	(mg/100 ml)	860
IgM	(mg/100 ml)	122
IgA	(mg/100 ml)	68
IgE	(u/ml)	87
C3	(mg/100 ml)	157

PHA Lymphocyte Transformation (dpm $[^{14}C]$ -thymidine)

Lymph	Serum	
Adult Reference	AB	25448
Adult Reference	Paediatric Control	24428
Paediatric Control	AB	32802
Paediatric Control	Paediatric Control	35290

CONTROL No. 5

Sex:	M
Age (months):	15
Weight (kg):	11,750
Expected weight for age (%):	110,2

Laboratory Results:

Total protein	(g/100 ml)	6,44
Albumin	(g/100 ml)	3,47
α_1 globulin	(g/100 ml)	0,21
α_2 globulin	(g/100 ml)	0,82
β globulin	(g/100 ml)	0,93
γ globulin	(g/100 ml)	1,00
IgG	(mg/100 ml)	960
IgM	(mg/100 ml)	115
IgA	(mg/100 ml)	59
IgE	(u/ml)	49
C3	(mg/100 ml)	172

PHA Lymphocyte Transformation (dpm $[^{14}C]$ -thymidine)

Lymph	Serum	
Adult Reference	AB	27026
Adult Reference	Paediatric Control	27444
Paediatric Control	AB	33593
Paediatric Control	Paediatric Control	34595

CONTROL No. 6

Sex:	M
Age (months):	16
Weight (kg):	9,220
Expected weight for age (%):	84,7

Laboratory Results:

Total protein	(g/100 ml)	7,12
Albumin	(g/100 ml)	3,69
α_1 globulin	(g/100 ml)	0,28
α_2 globulin	(g/100 ml)	0,88
β globulin	(g/100 ml)	0,88
γ globulin	(g/100 ml)	1,39
IgG	(mg/100 ml)	1446
IgM	(mg/100 ml)	171
IgA	(mg/100 ml)	67
IgE	(u/ml)	59
C3	(mg/100 ml)	106

PHA Lymphocyte Transformation (dpm $[^{14}C]$ -thymidine)

Lymph	Serum	
Adult Reference	AB	33144
Adult Reference	Paediatric Control	32457
Paediatric Control	AB	36726
Paediatric Control	Paediatric Control	30597

CONTROL No. 7

Sex:	F
Age (months):	20
Weight (kg):	13,870
Expected weight for age (%):	121,1

Laboratory Results:

Total protein	(g/100 ml)	6,97
Albumin	(g/100 ml)	3,70
α_1 globulin	(g/100 ml)	0,22
α_2 globulin	(g/100 ml)	0,85
β globulin	(g/100 ml)	1,04
γ globulin	(g/100 ml)	1,16
IgG	(mg/100 ml)	1084
IgM	(mg/100 ml)	147
IgA	(mg/100 ml)	114
IgE	(u/ml)	36
C3	(mg/100 ml)	168

PHA Lymphocyte Transformation (dpm $[^{14}C]$ -thymidine)

Lymph	Serum	
Adult Reference	AB	37395
Adult Reference	Paediatric Control	35637
Paediatric Control	AB	25494
Paediatric Control	Paediatric Control	26213

CONTROL No. 8

Sex:	F
Age (months):	22
Weight (kg):	11,600
Expected weight for age (%):	97,6

Laboratory Results:

Total protein	(g/100 ml)	6,17
Albumin	(g/100 ml)	3,27
α_1 globulin	(g/100 ml)	0,26
α_2 globulin	(g/100 ml)	0,84
β globulin	(g/100 ml)	0,77
γ globulin	(g/100 ml)	1,03
IgG	(mg/100 ml)	900
IgM	(mg/100 ml)	138
IgA	(mg/100 ml)	54
IgE	(u/ml)	166
C3	(mg/100 ml)	150

PHA Lymphocyte Transformation (dpm $[^{14}C]$ -thymidine)

Lymph	Serum	
Adult Reference	AB	29730
Adult Reference	Paediatric Control	24030
Paediatric Control	AB	36887
Paediatric Control	Paediatric Control	28460

CONTROL No. 9

Sex:	M
Age (months):	37
Weight (kg):	13,000
Expected weight for age (%):	87,5

Laboratory Results:

Total protein	(g/100 ml)	5,87
Albumin	(g/100 ml)	3,26
α_1 globulin	(g/100 ml)	0,20
α_2 globulin	(g/100 ml)	0,68
β globulin	(g/100 ml)	0,79
γ globulin	(g/100 ml)	0,94
IgG	(mg/100 ml)	1460
IgM	(mg/100 ml)	86
IgA	(mg/100 ml)	79
IgE	(u/ml)	10
C3	(mg/100 ml)	152

PHA Lymphocyte Transformation (dpm $[^{14}C]$ -thymidine)

Lymph	Serum	
Adult Reference	AB	38133
Adult Reference	Paediatric Control	34283
Paediatric Control	AB	58309
Paediatric Control	Paediatric Control	58804

CONTROL No. 10

Sex:	F
Age (months):	44
Weight (kg):	14,450
Expected weight for age (%):	92,3

Laboratory Results:

Total protein	(g/100 ml)	7,01
Albumin	(g/100 ml)	3,9
α_1 globulin	(g/100 ml)	0,28
α_2 globulin	(g/100 ml)	0,75
β globulin	(g/100 ml)	0,71
γ globulin	(g/100 ml)	1,38
IgG	(mg/100 ml)	922
IgM	(mg/100 ml)	200
IgA	(mg/100 ml)	135
IgE	(u/ml)	130
C3	(mg/100 ml)	139

PHA Lymphocyte Transformation (dpm $[^{14}C]$ -thymidine)

Lymph	Serum	
Adult Reference	AB	30206
Adult Reference	Paediatric Control	23872
Paediatric Control	AB	24190
Paediatric Control	Paediatric Control	23925

A. 3 TRYPAN BLUE STAIN FOR CELL VIABILITY (Phillips, 1973)

Two stock solutions were prepared:-

(1) A 2% solution of Trypan Blue (Sigma) in distilled water.

(2) A 0,3 M NaCl solution.

Equal volumes of the Trypan Blue solution and the saline solution were mixed just before use and added to the lymphocyte suspension in equal volume. The suspension was mixed well and allowed to stand at room temperature for 10 min. A wet preparation of the cell suspension was examined using phase contrast optics with a heat-absorbing filter, and 200 lymphocytes were counted. Lymphocytes taking up the blue stain were scored as non-viable cells.

A. 4 ESTERASE STAIN FOR IDENTIFICATION OF MONOCYTES

This stain is based on the methods of Yam, Li and Crosby (1971) and Li, Lam and Yam (1973).

The following reagents were prepared:-

(1) Buffered Formalin Acetone Fixative

Twenty milligrams of $Na_2HPO_4.2H_2O$ and 100 mg of KH_2PO_4 were dissolved in 30 ml of distilled H_2O plus 45 ml of acetone and 25 ml of formalin.

(2) Hexazotised Pararosanilin

One gram of Pararosanilin Hydrochloride was dissolved in 20 ml of distilled water and 5 ml of 0,1 N HCl were added. The solution was warmed gently in a 37°C incubator for 30 min, after which it was allowed to cool, filtered and stored in the dark. Two millilitres of a freshly prepared 4% $NaNO_2$ solution in distilled water was added to 2 ml of pararosanilin solution and shaken for 1 min. The colour changes to dark reddish brown.

(3) Alpha Naphthyl Acetate

100 mg of a naphthyl acetate was dissolved in 5 ml of 2-ethoxy-ethanol.

(4) 0,15 M Phosphate Buffer pH 7,6

2,38 g of $Na_2HPO_4.2H_2O$ was dissolved in 200 ml distilled water.
0,454 g of KH_2PO_4 was dissolved in 50 ml distilled water.
The KH_2PO_4 solution was added to the Na_2HPO_4 solution until the pH was 7,6.

(5) Incubation Medium

Fifty three comma four millilitres of the phosphate buffer, 3,6 ml of the hexazotised pararosanilin and 3,0 ml of the α naphthyl acetate solution were mixed in a beaker and the pH was adjusted to 6,1 with 1 N HCl. A red flocculent precipitate formed and the medium was clarified by filtration before use.

Staining Method

Blood smears or smears of lymphocyte preparations were air-dried and fixed for 30 secs in the buffered formalin acetone solution in a Coplin jar at 4°C. They were then immediately washed in three changes of distilled water and air-dried.

The fixed preparations were then placed in a Coplin jar containing the incubation medium for 45 min at room temperature and washed in three changes of distilled water. The slides were counterstained in a fresh 0,5% solution of methyl green dissolved in the 0,15 M phosphate buffer (pH 7,6) for 5 secs.

Lymphocytes stain a pale green colour, while cells with esterase activity such as monocytes or granulocytes stain a dark brick-red.

A. 5 CONSENT FORM

UNIVERSITY OF CAPE TOWN

DEPARTMENT OF PAEDIATRICS AND CHILD HEALTH

Date: . . . . . .

Name: . . . . . .

Mother of: . . . . . .

I hereby consent to my child being investigated by means of venipuncture and delayed hypersensitivity skin tests.

I realise these tests will not necessarily benefit my child directly, but that they may lead to a better understanding of other children's illnesses.

The nature and risks of the test have been explained to me by the doctor concerned.

Signed: . . . . . .

Witnesses: 1. . . . . (Doctor)

2. . . . . (Nurse)

A. 6 STATISTICAL METHODS

The statistical analyses of the results presented in this thesis were performed using standard methods as described in the following references:

Coefficient of variation, Student's T test and Correlation coefficient (Snedecor and Cochran, 1967);

Two- and three-way analysis of variance (Smart, 1970);

Friedman's two-way analysis of variance (Siegel, 1956).

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